

Simpler ways to good research degrees

The Royal Society has rebuffed the British government on its plan to reorganize the courses followed by graduate students, but both parties should be more concerned with undergraduate courses.

BRITAIN retains the distinction of enabling people to earn a research degree in the shortest possible time. Technically, a student can spend three years as an undergraduate and two years in putting together a thesis, emerging after five years as a fully fledged PhD, perhaps aged only 22. In many parts of the world, the United States and Germany for example, this is more commonly the length of time spent on a PhD course alone. Although such lightning progress towards a research qualification is now less common than formerly, the question remains of whether it can be wise.

The question has become an issue with the British government's plans for the reorganization (yet again) of British science, published a year ago. Among other things, it was proposed that for most students wishing to embark on a research degree, there should be a special year of study intercalated between undergraduate and PhD studies. But this year of study was intended to be a preparation for research that would be educationally valuable in itself, and which would earn the qualification of MRes.

For the research community, the rub came in the government's guess that research councils would in future spend more on MRes students and less on potential PhDs. Not unnaturally, the Royal Society set up a study group, under Professor Joe Vinen, to consider the proposal (see *Nature* 369, 91; 1994) and, in the light of the study group's report, rejected it as "educationally unnecessary". But the government and the research community are not so far apart as may appear. Indeed, what separates them is chiefly the now-common British question of who will pay for what, and how much.

By advocating the intercalated year and a three-year PhD course, the government has accepted that the normal education of a researcher should occupy seven years from university entrance to a PhD qualification. Vinen and his colleagues enthusiastically accept that implied promise, perhaps forgetting that it may be withdrawn if the government does not get its MRes. But the essence of their argument against the new proposal is that there is already such a variety of patterns of university science courses in Britain that it would be better to build on that than to introduce something entirely novel. Many undergraduate courses already last four years and the fashion is spreading. And there are many places at which the extra year could be fitted seamlessly onto the beginning of a spell of work towards a PhD.

The weakness of the Vinen argument is that it makes too great a virtue of diversity, much of which has arisen because

of the stratagems by which different universities have sought public funds to support longer undergraduate courses. Especially because British higher education can no longer rely on a supply of school-leavers with exactly the right specialist qualifications in science, the crying need is that every undergraduate science course should last for four years. In passing, it should give students a sense of the real world outside their institution. It should also give them an understanding of what research is like, and is about, for research is an essential element of science. A degree of uniformity is necessary. And it is worth fighting for on educational grounds.

The weakness of the government's case is that its extra year is a poor substitute for a uniform four-year undergraduate course. Its introduction could well be an intolerable distraction for serious students and an overly miscellaneous experience for others. The difficulty, of course, is that the Office of Science and Technology (OST), which is masterminding the reorganization of research, has no direct influence over undergraduate education, but can persuade research councils to pay stipends to graduate students, if inadequately. Vinen and his colleagues draw attention to a related anomaly; four-year courses are an extra expense for students or their parents, graduate courses an extra cost for the government. The Royal Society and OST should join forces to fight for properly financed four-year degrees.

Meanwhile, more attention should be paid to the needs of graduate students and of research. Part of Britain's present difficulty is that the output of PhDs exceeds the foreseeable demand for academics and that the slack is not fully taken up by industry. That, of course, is both a measure of British industry's shortsightedness and a cause of British industry's poor economic performance: it employs too little skill. The British government should be seeking to remedy that state of affairs. Then it would find that young people are again enthusiastic at the prospect of a life in science. □

Anglo-French dispute

European air transport is a long way from being the free market on which the European Union has set its heart.

As recently as a century ago, either Britain or France would have declared war on the other last Monday. The issue that divides the two governments is accurately reminiscent of classical *casus belli*, that of whether transport vehicles



carrying people bent on peaceful purposes can enjoy the right of legal free passage. A century ago, of course, sea passage (through the Dardanelles, for example) was at the centre of these disputes. Aircraft have now taken the place of ships, but the contentiousness of disputed rights of passage has not abated. Last Monday, British Airways planned to land an aircraft at Orly airport, in south-suburban Paris, in defiance of a French declaration that it would not be allowed to land. Confrontation was avoided (and the disputed flight postponed until not later than the end of June) only after the respective transport ministers spent the weekend on the telephone to each other.

In reality, this dispute is not so much a test of the belligerence of the governments of Britain and France as of the resolution of the European Union (EU) in its pursuit of free competition. Not so long ago, ten out of the 12 present members of the EU maintained a national passenger airline to carry their flags to distant parts of the world, but also to parlay landing rights for their aircraft from governments elsewhere. The costs of these programmes were huge; they were met partly by European air travellers, who paid (and still pay) some of the highest air fares in the world, and partly by the same people in their role as taxpayers, who made possible the direct subsidy of European airlines. The dispute between Britain and France this week arises from the European Commission's efforts to make Europe's airline industry more competitive.

The nature of the dispute shows how much there is still to do. A truly competitive European airline business would be simply organized; there would be a number of airlines owned privately, by shareholders, strict regulation of safety and traffic by a transnational authority and some arrangement for allocating landing slots at European airports to those who would make the best use of them (perhaps by annual auctions).

The commission is struggling towards that goal, but only slowly. In the Anglo-French dispute, it has merely concluded that Orly airport, now used exclusively by aircraft serving French domestic routes, is an anti-competitive French fiefdom, and has awarded British aircraft access (as well as the right to carry passengers onwards to Lyons and Marseilles). Three British airlines are anxious to get started. Subplots in the dispute include British resentment (matched elsewhere in Europe) of a French plan to increase the capital of nationally owned Air France in a manner that will be indistinguishable from a subsidy and French demands for more landing slots at London airports.

It is some comfort to know that there will not now be a shooting war, but the first lesson to be learned from this affair is that it is absurd that the EU has so engineered matters that it is involved in the details of which aircraft can land, or fly to, where. It does not have the power to insist that nationalized airlines should be denationalized, but it can forbid subsidies absolutely, and should do so. Similarly, it can (as the Orly case shows) intervene on airport access, but should go the whole hog and devise a Europe-wide system for allocating landing slots to airlines able to make good use of them. Then it should sit back and watch Europe's airline industry become efficient. □

Back to Bretton Woods

A scheme for reintroducing fixed international exchange rates is unlikely to succeed in the near future.

Mr Paul Volcker, the chairman of the Federal Reserve Board (or central bank) in the United States, is plainly an ambitious man: according to *The Wall Street Journal* last week, he is leading a group of central bankers seeking a way back to Bretton Woods, the system of fixed exchange rates the Western world devised in the closing months of the Second World War. The arrangement served the international community reasonably well until it was abandoned in 1971, largely because of the inability of the United States to keep the US dollar strong and to fight the Vietnam War at the same time. But Volcker's ambition is not easily distinguished from nostalgia, and is unlikely to be satisfied.

By all accounts, the Volcker group has already concluded that bringing back Bretton Woods would require of all participating governments a remarkable degree of financial discipline. They would have to keep their budgets nearly in balance and reduce their accumulated debts so that fiscal measures (taxation) could again become effective regulators of their economies. The control of inflation would similarly be a central objective of policy, while, in due course, 'economic convergence' would become central. Volcker, of course, has rediscovered what the members of the European Union argued out at Maastricht at the end of 1992, the celebrated preconditions for a common European currency. The events of 1993, when Britain and Italy abruptly abandoned their fixed exchange rates, are a vivid illustration of what can go wrong with such arrangements.

Yet the nostalgia is understandable. There is little doubt that a large part of the impetus for the growth of world trade in the decades immediately after the Second World War was that governments and traders knew what their money was worth (barring the occasional traumatic devaluation). It is less pleasing that, by excluding the developing countries, Bretton Woods deprived them of a fair share in that growth, as well as of the financial assistance of organizations such as the International Monetary Fund. Now there are even bigger problems to be tackled, the inclusion of Russia, the republics and Central Europe in a unified system for example. And the world's currency traders have become skilled at telling when a currency is being supported at an artificial level, to their own great profit.

None of this implies that Volcker's enterprise should be discouraged. On the contrary, it will be valuable if central bankers are induced again to discover for themselves that the simplest way of giving the world sound money is to run it as if it were a unified place, with common goals and common policies for achieving them. But the place to start is with the here and now, with the continuing need for technical and financial assistance in Europe east of the Elbe, with the need for an understanding between the industrial West and China and with the need for prosperity in the developing world. □

MIT observatory in last-ditch appeal over withdrawal of NSF funding

Boston. Astronomers at the Haystack Observatory in Westford, Massachusetts, will later this month make a last-ditch appeal to the US National Science Foundation (NSF) to reverse a decision to cut all funding for the telescope in four years' time.

Despite a recent three-year, \$1.5-million upgrade, the NSF announced in March that it was cutting funding for the observatory, a research facility of the Massachusetts Institute of Technology, by about 10 per cent, down to about \$1.1 million.

The future looks even grimmer. Although the NSF has pledged continued support for Haystack's group in very long baseline interferometry (VLBI), it plans to "ramp down" support for the 37-metre radio-telescope over the next three years, terminating it completely after fiscal year 1997.

The decision has left Haystack personnel reeling, particularly as the renovations mean that it can now operate at frequencies 10 times higher than originally planned. According to Richard Barvainis, an astronomer at Haystack, the latest improvements make it the largest single-dish telescope in the United States operating at 3 millimetres, and the most sensitive in this wavelength band for studying small-diameter sources such as stars and molecular clouds.

Ironically, the NSF decision has come shortly after its officials praised Haystack

staff for doing "a superb technical job" with the upgrade. "They not only paid for this work, they also pushed us in this direction," says Joseph Salah, director of the facility. "Now they won't give us a chance to show what the revamped telescope can do."

But the cutbacks were not entirely unexpected. Last year the NSF commissioned a report from a panel led by Joseph Taylor of Princeton University, asking it to evaluate five university-based observatories, concentrating on millimetre-wavelength radio astronomy. Haystack was rated last, even though the VLBI group was praised, and the recent improvements described as "a substantial technical achievement".

Part of the problem is that Haystack is 30 years old and located close to sea level on the East Coast. "There are limitations as to what you can do with an older telescope in this location," Salah admits. "We're not on top of Mauna Kea."

Yet he and his colleagues also believe that the timing of the NSF review put them at a serious disadvantage, as they had just finished engineering tests after the upgrade, and had no new scientific results to show.

Haystack scientists are not yet throwing in the towel. They see their three-year budget proposal, due at NSF by the end of this



Haystack awaits its fate.

month, as a chance for an appeal. Ways of trimming costs and streamlining operations will be presented. (Jobs have already been lost as a result of the budget cuts, and additional redundancies seem inevitable.)

"We want to emphasize the scientific results obtained since the upgrade and our plans for exploiting the new capability in the future," Salah explains.

Philip Myers, an astronomer at the Harvard-Smithsonian Center for Astrophysics (CFA) and a long-time subscriber at Haystack, believes the telescope can play a "useful role in helping astronomers to solve a long-standing puzzle — how stars form. Most of our understanding of this process is based on inferences rather than direct observations. Haystack has the combination of spatial and spectral resolution needed for the job."

Salah's strategy is to "let the science speak for itself. If we can't make a good scientific case, so be it." He is determined to keep the observatory open, no matter what NSF decides three years from now. The Air Force and NASA already contribute nearly \$5 million of the \$6-million annual budget, and those sources seem secure for now.

But Hugh van Horn, NSF's astronomy director, is not too encouraging. "This is not a criticism of the science being conducted at Haystack," he says. "The problem is one of limited resources. We need to shift some of those resources to maximize benefits to US astronomy as a whole."

In principle, notes Patrick Thaddeus of the CFA, the concept of pruning commitments is widely accepted. "These days, that's how science is done," he says. "Of course, everyone accepts this idea until they're told that their facility is the one facing the axe."

Steve Nadis

Maths teachers 'would welcome checks'

Washington. University mathematics departments should take stronger steps to evaluate the quality of teaching by faculty members, and reform pay and promotion structures in a way that rewards good teaching, according to the main US mathematicians' professional societies.

An investigation by the Joint Policy Board for Mathematics — a joint forum of the American Mathematical Society, the Mathematical Association of America and the Society for Industrial and Applied Mathematics — found that, contrary to popular belief, university mathematicians would welcome such changes.

Richard Herman of the University of Maryland, the chairman of the board, says that mathematics departments in public universities should reform themselves to acknowledge the importance of teaching before impatient state governments force them to do so. Herman called on mathematics departments to "think of this as an opportunity" for constructive reform.

The board visited 26 mathematics de-

partments and surveyed 2,000 faculty members, and found widespread dissatisfaction about the lack of rewards for teaching. Its report, *Recognition and Rewards in the Mathematical Sciences*, falls short of recommending that teaching should be rewarded on a par with research, which was by far the predominant performance measure in the departments visited.

In a carefully worded passage, it says that "research should, in general, continue to have a maximal status in the rewards structure". But it calls for a "broader and more flexible" structure that also takes into account teaching, interdisciplinary research and other work in giving promotions and pay increases.

But the report finds deep scepticism about the most widely used means of teacher evaluation, namely the student questionnaire. Mathematics lecturers, it appears, are fed up taking the rap when such surveys place them in a bad light next to their counterparts teaching arts and social science subjects.

Colin Macilwain

Japan optimistic on moves to resume commercial whaling

Tokyo. Japan's whaling fleet may emerge as both a winner and a loser in its fight to resume commercial whaling at next week's meeting of the International Whaling Commission (IWC) in Mexico.

After years of debate and scientific analysis, the plenary session of the IWC is expected to accept in principle a revised management procedure (RMP) that has been devised by the IWC scientific committee to allow resumed commercial whaling for the relatively abundant minke whale.

At the same time, however, there is a strong possibility that a French proposal to establish a sanctuary in the Southern Ocean will be accepted by the IWC, thus preventing Japan from whaling in the Antarctic, where its commercial whaling fleets used to operate. On a separate front, Japan is making a new move to win IWC approval to start research whaling for minke whales in the North Pacific.

The IWC introduced a moratorium on commercial whaling in 1986, on the grounds that there was insufficient data on the size of whale populations and reproduction rates to know whether commercial whaling could be sustained.

By 1991, the commission's scientific committee had developed the RMP, and estimated the population of Antarctic minke whale at 760,000, well above the number required to resume commercial whaling (see *Nature* 357, 532; 1992).

The representatives of most of the countries represented on the IWC were reluctant to adopt the RMP — and thus to lift the moratorium — because they feared it would be politically unacceptable at home. Instead, they asked the scientific committee to address further questions about the management procedure (see *Nature* 363, 9; 1993).

But the US administration of President Bill Clinton is now pressing for acceptance of the RMP in Mexico, and three leading environmental organizations — Greenpeace, the World Wide Fund for Nature, and the International Fund for Animal Welfare — are not opposing this move.

The environmental groups fear that any further delays could lead to the break-up of the IWC, resulting in the whaling nations walking out to resume commercial activities. They will, however, press for stringent safeguards that will, if implemented, delay resumption of commercial whaling for several years.

Furthermore, there is a strong possibility that a French proposal to establish a whale sanctuary in the Antarctic will be adopted at the meeting. But this will not please the Japanese; Fukuzo Nagasaki, director-

general of Japan's Institute of Cetacean Research says he is "pessimistic" about the vote on the sanctuary.

Whaling nations need the support of at least one quarter of the 30 or so IWC members attending at Mexico to block the sanctuary proposal. At present, however, Japan can only count on six or seven votes.

Nagasaki says that the critics of whaling have created a "double net" with their sanctuary proposal so that, even if the RMP is

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Whale meat still on the menu.

accepted and the moratorium is lifted, Japan will still be unable to resume commercial whaling in the Antarctic.

Japan has made a new move by suggesting to the IWC scientific committee prior to the plenary session that it should be allowed to catch 100 minke whales in the North Pacific for research purposes. Until now, Japan's research whaling has been confined to the Antarctic.

According to Nagasaki, the Antarctic research aims at determining population size and reproduction rates in minke whales. In contrast, research in the North Pacific will try to identify the precise size and distribution of breeding stocks of minke whales.

Such data, which will include DNA analysis of the whales, is critical for operation of the RMP, he says. If stock sizes are large, then the sustainable quota under the RMP is also large; but if the whales are split into small groups, the quotas may be too small to make commercial operations viable.

Nagasaki says the North Pacific proposal is not a strategy to get around the attempts of critics to block whaling in the Antarctic. But he says it is indirectly related to Japan's attempts to get IWC permission for small whaling communities in Japan to catch minke whale in the Pacific.

The Royal Society for the Protection of Animals, one of the UK's leading animal welfare organizations, condemns the proposals to resume both scientific and commercial whaling — even on a small scale — in protest at the methods currently used to kill whales.

David Swinbanks

Weather satellite decision 'epitomizes new US strategy'

Washington. US military and non-military weather satellite systems are to be combined in a move that officials of the Clinton administration claim will save the government \$300 million over the next five years, and up to \$1.5 billion after that.

The restructuring was announced last week by John Gibbons, the president's science and technology adviser, who said that it is being undertaken as part of Vice-President Al Gore's mission to "re-invent" government.

The move will combine existing polar-orbiting weather satellite systems, at present operated separately by the Department of Defense and the Department of Commerce's National Oceanic and Atmospheric Administration (NOAA), into a single programme led by the latter. The National Aeronautics and Space Administration (NASA) will carry out research and development on future enhancements to the combined programme.

The administration also announced that it intends to proceed with the Landsat remote sensing programme by building Landsat 7 under another new interagency structure. Landsat 6 was lost on launch last year. NASA will take over the acquisition of the new programme from the Department of Defense, the commerce department will operate the satellite, and the US Geological Survey — part of the Department of the Interior — will run the archive storing Landsat data.

According to Gibbons, these moves reflect improved collaboration between government agencies under the new National Science and Technology Council (NSTC), which the president set up last autumn (see *Nature* 365, 195; 1993).

Under the executive order that covers the combined weather satellite programme, Defense, Commerce and NASA will establish an Integrated Program Office to run the new programme, which will consist of three satellites instead of the existing four (two civilian and two military).

The three agencies will pursue negotiations with the European Eumetsat weather satellite programme about incorporating a European satellite into the converged system. Officials say this could save even more money, but admit that the national security role of the new US programme may make collaboration more difficult. "National security is seen as absolutely supreme," one official said.

Vice-President Gore said in a statement that the move "epitomizes the spirit and potential of reinventing government". Gibbons said the merger now being achieved had been unsuccessfully attempted eight times in the past 20 years. **Colin Macilwain**

HIV health workers 'need better protection'

Paris. A group of French researchers, physicians, and healthcare workers have created an association to seek improved protection for healthcare workers from the risks of becoming contaminated with human immunodeficiency virus (HIV) through hospital accidents.

Fresh in their memories are the mid-1980s, when scientists and physicians failed to anticipate the danger of donated blood contaminated with HIV. Francis Cheilan, a vascular surgeon at the Passy Hospital near Paris, and president of the *'Union pour la Protection des Soignants et de leurs Patients'**, is keen that history should not repeat itself.

In particular, the association wants the health authorities to make it obligatory for hospitals to introduce precautions to reduce the risk of accidental contamination by HIV. Use of self-sheathing needles, for example, could reduce needleprick accidents by two-thirds, says Cheilan. He also points out that such precautions would simultaneously protect healthcare workers from hepatitis B and C and other bloodborne diseases.

Similar precautions were also recommended by the ministry of health in a report released two years ago. But only a few hospitals have introduced safer procedures, says Cheilan. He claims that efforts by the health authorities to save centimes — a self-sheathing needle costs FF3.0, whereas a standard one costs FF0.8 — could cost lives, and accuses them of erecting a "wall of silence" for fear of creating panic.

Part of the problem, says Cheilan, is that the authorities are not convinced of the risks. Yet around 30 healthcare workers have already been confirmed as contracting HIV in work accidents in France, according to the association. It also claims that the real figure is probably closer to 300, and will probably climb further as seropositive patients become more common.

In a bid to obtain better estimates, several Paris hospitals recently began testing simplified procedures for declaring work accidents. This was a response to the association's claim that existing procedures are so long and complicated that many healthcare workers instead prefer to cross their fingers.

The association believes that better social protection for contaminated healthcare workers is an essential step towards creating a climate in which the problem can be openly addressed. It is therefore demanding specific legislation allowing healthcare workers to claim adequate compensation.

At present, workers contaminated by HIV come under the legislation that covers general work accidents. This only entitles them to an invalidity pension, which is around one-third of salary, and many young healthcare workers are already only on the minimum wage.

The government introduced regulations last year linking the level of invalidity pension to the blood count of CD4 T lymphocytes (the cells depleted in AIDS). But it overlooked the fact that the legislation covering these regulations only allows invalidity pensions to be revised once every five years, irrespective of whether the worker gets sicker before the end of this period.

The association is urging the National Assembly to correct this contradiction immediately. Moreover, the decree formally stated that accidental HIV contamination comes under the law on work accidents. Cynically, say some, this means that healthcare workers are forbidden from seeking further compensation for hardship.

Pointing out that people contaminated by HIV through blood transfusion are auto-

matically entitled to substantial compensation for "hardship caused", the association earlier this year wrote to Simone Veil, the health minister, demanding legislation to provide staff working in the front line with compensation more appropriate to the consequences of HIV infection.

Cheilan claims that the government and health authorities are "completely ignoring" the problem of accidental contamination of healthcare workers. In reply to a parliamentary question on the matter last December, Veil said the government had "a strong policy of information and training." But the association wants more done, and is considering taking a case to court to attract further attention.

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Greeks rue the fruits of success

Athens. The Greek government is expected to approve this week the creation of a new unit to monitor the way in which public funds are spent on research, and to evaluate the performance of individual institutes.

But an overall shortage of money means that scientists and research groups receiving a good evaluation are unlikely to be rewarded with extra funding, while the government is not expected to accept the political price of closing institutes that perform badly.

Many Greek scientists have long been concerned by the lack of a proper system of scientific evaluation of their work. Some research institutes have installed their own systems, but many have not.

In 1987, Eleftherios Economou, then the country's secretary general for research, introduced a complex formula for assessing government-funded research institutes and deciding how much money they should receive. The system took into account not only publication records, but also the amount of money raised from external sources — and a general judgement of scientific achievement made by the secretariat.

But the system has not been used in the 1990s. The real value of government funding has dropped steadily, and it now barely covers salaries and basic running costs. Research institutes that are successful in attracting outside funding end up finding their public funds reduced, as the government feels they are less in need of public support.

The Foundation for Research and Technology Hellas (FORTH), for example, which runs seven research institutes, four of them in Crete, has seen its government funding fall from Drs1,176 million (US\$4.6 million) in 1990 to Drs614 million in 1993.

Economou is now FORTH's director, and criticizes the government's policy, as he feels it fails to provide any long-term security for his research centre, the second largest in Greece. He is seeking a more solid basis of government support, based on his centre's scientific track-record.

Many scientists feel that the judgement of the general secretariat now carries more weight than was originally intended by Economou, and that a close working relationship between the institutes and the secretariat has become more important than the scientific value of the institute's research.

It is in response to these concerns that the new unit is being established. Nicos Christodoulakis, the secretary general, has called a meeting on 20 May of institute directors to discuss the mechanisms by which it will assess institutes.

But whatever the new assessment criteria, it is clear that reward for good performance will not be financial. Christodoulakis says that simply publishing the results of the assessment exercise should be sufficient to stimulate researchers to improve their own output by encouraging greater competition.

Many Greek scientists welcome the prospects of stronger central evaluation. Socrates Tzartos, head of biochemistry at the Hellenic Pasteur Institute in Athens, for example, accepts that publishing the results of the evaluation may be effective. But Tzartos and others are sceptical about the government's ability to put its plans into practice.

Allison Abbott



Economou: seeks a better reward.

Mixed reaction to Mandela's appointments

Cape Town. Pre-election hopes that science would be given its own government department in South Africa were partly fulfilled last week when Nelson Mandela, the country's first democratically elected president, announced the creation of a Ministry for Arts, Culture, Science and Technology in his new cabinet.

But enthusiasm for this initiative has been tempered by the appointment of Ben Ngubane, a political supporter of Chief Mangosuthu Buthelezi, as the responsible minister, and of Mandela's estranged wife Winnie Mandela as deputy. "Their lack of experience in arts, culture, science or technology is one of the few things they have in common," commented the *Cape Times*.

Ngubane is a mild-mannered 52-year-old physician and ardent Zulu nationalist. He was Minister of Health in the former self-governing territory of Kwazulu, and chief negotiator for Buthelezi's Inkatha party in the constitutional negotiations. His appointment reflects the provisions of the interim constitution, which required Inkatha be given three cabinet positions, as it received a surprisingly large 10 per cent of the national vote in the elections.

Winnie Mandela, 59, was formerly a social worker, and headed the welfare department of the African National Congress (ANC) before resigning two years ago following her separation from her husband. She herself has been reported in the media as describing her appointment as "weird".

One thing is certain. With her flair for controversy — and unless responsibility is

specifically allocated to Ngubane — science and technology will not be out of the political limelight. "We've always said that what science and technology needs is a powerful advocate in government," quipped an official.

The cabinet appointments suggest that the new ministry was seen by the ANC as one over which it could afford to relinquish control.

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Winnie Mandela:
high profile.

is the *alma mater* of both Nelson Mandela and Buthelezi.

The 60-year-old Bengu has a doctorate in international relations from the University of Geneva, and wide experience at different levels of education, having worked both as a school-teacher and a training-college lecturer in Natal, and as a professor at the University of Zululand. For 12 years

Peter Brooke/Rea Features

The academic world has seen two surprise appointments in the new government. The new Minister of Education is Sibusiso Bengu, rector of the University of Fort Hare. Located in the Eastern Cape town of Alice, it is

he was secretary for research and social action at the World Lutheran Federation in Switzerland.

Bengu says that his first priority is to establish a single system of education for the country. Mandela is due to announce shortly that all 18 existing education departments will fall under the new ministry, which will then be responsible for integrating personnel from the existing departments into one national and nine provincial departments.

Bengu says that he expects to appoint a new director-general within a week. One of the most likely candidates, however, Jakes Gerwel, rector of the University of the Western Cape, has already been snapped up by Mandela to fill this post in his own office.

The new minister of health is Nkosazana Dlamini, 45, a researcher with the Medical Research Council (MRC). An exile who qualified as a physician at the University of Bristol in the United Kingdom, Dlamini returned to join the MRC's Durban centre in 1991, where she has been working on AIDS and maternal and child health.

South Africa's re-admittance to the World Health Organization earlier this month should ensure the support of the international community in implementing new programmes. More importantly, it signals an end to isolation from international scientific bodies.

Michael Cherry

Russia is urged to lift science spending

Moscow. Boris Saltykov, the Russian science minister, has proposed that the government commit itself to spending a minimum of four per cent of the state budget on research. Although considerably less than the level of funding enjoyed in the mid-1980s, it is still higher than the 3 per cent of the budget which science received last year.

The proposal was made as part of a draft law on science and technology policy presented to the government Presidium earlier this month. Among its supporters was Yuri Yarov, the deputy prime minister, who said it was "vital" to fix a minimum level of support for science, as a further reduction in spending would have major consequences.

The Ministry of Finance, however, is reported to have opposed writing any specific targets into the new law, which is planned to specify the roles of Russia's main science funding agencies. A revised version is due to be re-submitted to the Presidium next month. □

Scientists join the funding queue

Cape Town. The most important task facing South Africa's new Ministry of Arts, Culture, Science and Technology, according to many of the country's scientists, is to persuade the government to increase the research budget in the face of its many other spending priorities.

Preliminary allocations for this year's budget total R787 million (US\$222 million), only 4 per cent higher than last year at a time when inflation is running at 9 per cent. Furthermore, although the budget was approved by the Transitional Executive Council in March, it could still be amended before it is tabled in parliament next month.

The most significant feature of this year's budget is that additional funds have been allocated to the government's major funding agency, the Foundation for Research Development. This is the body that supports research at universities, 'technicons' and museums, and runs three national research facilities. It is proposed to increase its budget by 23 per cent to R143 million, R16 million of this being earmarked for replacing obsolete equipment.

The proposed allocation to the Medical Research Council has increased by 3.5 per cent to R48 million, and that to the Human Sciences Research Council by 6 per cent to

R81 million. Both agencies spend a relatively large proportion of their funds on university research groups and other external laboratories.

In contrast, allocations to the three councils with little or no agency functions will decrease as a proportion of the total budget, although continuing to make up by far the greatest portion of it.

Funding for the Council for Scientific and Industrial Research, which conducts in-house applied research, will grow by only one per cent, to R233 million. The budget of the Agricultural Research Council has decreased by 0.5 per cent to R220 million, and that of the Minerals Technology Council by 5 per cent, to R61 million.

This is the last research budget to be allocated on the present system, under which a cabinet committee made up of the ministers of education, health, trade and industry, and mineral and energy affairs divides up the budget after taking secret advice from the Scientific Advisory Council.

The entire research budget is likely to become the responsibility of the new council from next year. One of Ngubane's first tasks will be to appoint a new advisory council, as the term of office of the present council expires at the end of June. **M. C.**

Business professor takes post of science minister in Italy

London. Italian science is likely to see increasing priority being given to links between the academic and industrial worlds following the appointment of Stefano Bodesta, a prominent professor of business management, as the country's new minister of universities and research.

Bodesta is currently director of a centre which carries out research into industrial and business organization at the private Luigi Bocconi University in Milan — one of the country's leading business schools.

Together with several other members of the university's faculty, he has been involved in the creation of Forza Italia (Freedom Alliance), the political party set up by Silvio Berlusconi, the industrial magnate.

Berlusconi was last week appointed the country's prime minister following his party's successes in last month's general election. Bodesta is seen as one of several 'technocrats' who have been brought into government by Berlusconi in an attempt to resolve Italy's chronic economic difficulties.

The 55-year-old Bodesta is little known in the scientific community. But he has considerable experience of the academic world, having previously taught at the universities of Parma, Milan and Venice. He is the author of a number of books on topics such as strategic decision-making and the theory of industrial economics, and has been on the faculty of the Bocconi University since 1982.

One of the tasks facing the new government is to decide how far it is prepared to go in supporting proposals for funding research over the next three years that were proposed to Parliament shortly before the election by the departing minister of universities and research, Umberto Colombo.

Under Colombo's plans, the overall research budget was to grow by a massive 35 per cent over the two years 1994 to 1996, with particular increases in support for areas of applied research, destined to rise from 100 billion lire in 1994 to 1,000 billion lire. Direct government funding for university research was also due to rise sharply, from 250 to 450 billion lire, in this period.

Colombo's stated target was to increase Italy's spending on research and development from its current level of 1.6 per cent of gross national product to 2.0 per cent by 1995. Given the country's current economic problems, funding increases of the magnitude proposed by Colombo are unlikely to materialize. But there is a good chance that, under Bodesta, pressure will be maintained by the new government for some of the organizational changes recommended by Colombo for improving the efficiency with which research funds are used. □

US motor giants fail to quell enthusiasm for electric cars

Washington. The oil and automobile industries have failed in a last-minute effort to stall a tough Californian regulation that will require all car companies operating in the state to develop and sell electric cars by 1998.

After a two-day review meeting in Los Angeles last week, the Californian Air Resources Board endorsed a plan to require car companies operating in the state to ensure that, by 1998, two per cent of sales are of cars emitting no pollutants — electric cars, in effect.

The mandate is expected to have a wide impact, as no major Japanese or US car maker can afford to exclude itself from the Californian market. All will have to start mass production of at least one electric car in their range to comply.

The board also rejected a proposal that it should review the mandate every six months, rather than every two years as it had proposed. It said that such a change would effectively paralyse investment in electric car technology.

The only potential barrier remaining is



the possibility that Pete Wilson, the conservative California governor, who is thought to oppose the mandate, may, if re-elected this autumn, sack the Air Resources Board and have the mandate lifted. But so far he has refrained from a move that would deeply alienate the strong Californian green vote in an election year.

The state of California pioneered air pollution law, and federal legislation therefore gives the state special powers to set its own laws and mandates on vehicle emissions. All other states work to rules laid down by the Environmental Protection Agency (EPA) in Washington. Many northeastern states have already petitioned the EPA to set a mandate in line with California's, and the outcome of last week's meeting will increase the pressure.

As well as addressing local air pollution problems, the mandate is intended to reduce greenhouse gases, one-third of which come from transport. But electric cars will help to

alleviate the greenhouse effect only if the electricity to power them is produced without pollution: if it is generated with oil or coal, an electric car may produce more greenhouse gas than a petrol driven one.

"The global warming impact may be almost zero, depending on how the electricity is made," says John Houghton of the UK Rutherford Appleton Laboratory, one of the world's leading experts on the greenhouse effect.

Given the costs and complexities of existing battery technology, the 1998 deadline may well prove to be over-optimistic. But the record of the big-three US car manufacturers — Ford, Chrysler and General Motors — of relentlessly opposing pollution and safety mandates in the past has undermined the credibility of their opposition to this one. Their proposal that California should turn to the National Academy of Sciences for advice was ignored by the board.

The United States Advanced Battery Consortium, a high-powered research and development collaboration led by the big-three car manufacturers, also suffered a rebuff. It told the board that the mandate had come too early, and would only lock car companies into immature and expensive battery technology. But other battery developers said they could mass produce batteries at reasonable cost if the mandate was there to guarantee them a market.

One impetus behind the mandate has been the emergence of a number of small companies promising to pioneer electric car technology and create a world-beating industry in California.

The debate over the mandate has to some extent developed into a war of words (and cultures) between these companies and the big-three manufacturers from Detroit.

The state's decision to press ahead may oblige the big-three to reconsider their opposition and prepare to comply with the mandate. Green activists are already suggesting that General Motors is ready to move swiftly to do so, perhaps by coming to an agreement with a Californian company, Electricar of Sebastopol, to modify part of the output of the Californian small car factory that GM runs jointly with Toyota.

Colin Macilwain

● A conservative Member of Parliament has been elected as president of the UK's Electric Vehicle Association in a bid to stimulate political support for electric cars in Britain. Andrew Robathan, MP for Blaby in Leicestershire, demonstrated his commitment to such vehicles last week by driving one round the forecourt of the offices of the Electricity Association, close to the Houses of Parliament.

Neuropsychologist's death sparks enquiries

Montreal. McGill University in Montreal has set up three separate enquiries into the circumstances surrounding the suicide of a prominent neuropsychologist. Her death followed the revelation that she had been given a reprimand — which she was continuing to contest — for failing to observe the university's regulations on the use of human subjects in experiments.

Justine Sergent, an associate professor in the Montreal Neurological Institute, an affiliate of the university, was found dead last month together with her husband, Yves Sergent, after the publication of an article referring to her reprimand appeared in *The Montreal Gazette*.

In a letter sent shortly before her death to a number of faculty members at McGill, Sergent had claimed that she had been locked in a continuing struggle with other members of her institute over a number of years, and that the referral of her activities to the ethics committee's had been motivated by professional jealousy. But the latter charge has been strongly rejected by university officials. In a memorandum to staff and students, Richard Murphy, the head of the institute, says that allegations that the committee acted for other than ethical reasons were "completely untrue".

"In dealing with an extremely difficult situation, our ethics committee was meticulous," wrote Murphy. "Its decisions were based on the facts, and it has followed its mandate to protect the interests of the volunteers that participate in our research."

Sergent had been working on the origins

of the functional lateralization of the brain, using positron emission tomography (PET) to record patterns of activity within the brains of human subjects. She had published in a number of prominent journals, including both *Science* and *Nature*.

Some of her results had been contested by other members of the institute. In 1992, for example, she and two colleagues published a report in *Science* indicating, among other results, that she had not been able to find a response to musical melodies in a particular region of the brain.

A paper published last month by other members of the institute reports such a response, and suggested that Sergent's failure to observe a response had resulted from inappropriate control conditions (*Journal of Neuroscience*, 14(4), 1908–1919; 1994).

Her reprimand by the university followed her use of PET techniques for testing subjects' reactions to stimuli provided by musical notation. Sergent had received the approval of the ethics committee for experiments using human faces as visual stimuli. But she went on to use the same technique for testing reactions to musical notation, and believed the ethics committee's approval covered that experiment as well.

Sergent argued in her defence that her subjects gave fully informed consent to being tested, and that their health was not compromised in any way, with which McGill concurs. But, following an inquiry by the ethics committee, she was reprimanded by the principal of the university in January 1993 for what the university has described

as "academic misconduct".

The reprimand became public as the result of an anonymous letter sent to the *Gazette* — as well as to senior officials in the university, the Medical Research Council and another funding agency — which claimed she had built her scientific career on the basis of what colleagues at McGill had long suspected was scientific fraud. (None of her collaborators was implicated in any way.)

The *Gazette* learned about the reprimand from a statement sent to the newspaper by the university in response to the letter and its allegations. Its subsequent article was published after an interview with university representatives and with Sergent in which the newspaper's editor later said both had described their positions "coherently and without rancour".

In a subsequent letter published by the newspaper, a group of Montreal neuropsychologists and other scientists expressed concern that it had published allegations of scientific fraud "without waiting for the results of an official investigation". They described Sergent as "among the most productive neuropsychologists in Canada".

In a separate article, Peter T. Fox, director of the research-imaging centre at the University of Texas Health Science Center in San Antonio, Texas, claimed that Sergent had been a casualty of both her own "uncompromising standards of excellence" and of an "inhospitable work environment".

He described allegations that Sergent, who had been a close personal friend as well as a scientific colleague, might have committed a scientific fraud as "a deliberate and outrageous falsehood". He also claimed that Sergent found herself under pressure as a woman in a field that remains male-dominated, and described her PhD degree in psychology as an anomaly in a medical department which "could only have made her situation less certain and more stressful".

The university has rejected Fox's implication that the judgement of the ethics committee was based on issues other than ethical concerns. But, in response to a request from Sergent — who had hired a lawyer to defend herself against the university's charges — the university is carrying out a scientific audit of her work.

This is being conducted by Pierre Bois, a past president of the Medical Research Council, and Marcus Raichle of Washington University in St Louis, Missouri, a respected authority on PET.

A separate inquiry will be conducted by Caspar Bloom, president of the Montreal bar, into McGill's internal procedures and the way that these were applied to Sergent's case. The university is also trying to find out who sent the original allegations of scientific misconduct to the newspaper.

David Spurgeon

Clementine's 'not gone forever'

The Clementine spacecraft — which captured this image of the Earth on its Moon visit last month — had already proved itself before an engine misfiring sent it into a 70 r.p.m. spin, its supporters say.

The craft — a low-cost offshoot of the Department of Defense's now-defunct Star Wars programme (*Nature* 367, 207; 1994) — had its mishap ten days ago. Coming immediately after a blaze of publicity which favourably compared the \$80 million mission to more expensive space projects, this no doubt brought quiet satisfaction to some dull lives at NASA headquarters.

But Stewart Nozette, deputy manager of the Clementine programme, says its primary goals are already being met and that the technology has proved itself. After weekend tests, he reports that the craft will "definitely get to a stable situation." Mission control hopes to slow the spin to 20 r.p.m. and then decide whether to send Clementine on to the asteroid Geographos, as originally planned.

C. M.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

BMCO/SP1

'User' links strengthened in NERC shake-up

London. Britain's Natural Environment Research Council (NERC) has decided to eliminate the three senior positions in its current management structure responsible for running separate science-based directorates, and to replace them with a single director for science and technology.

The move is designed to improve both the management efficiency of the NERC and its responsiveness to the needs of its 'user' communities. But it has also highlighted ambiguity over whether, following last year's white paper (government policy document) on science, the research council is itself an independent 'contractor' of research, or a 'customer' acting on behalf of government and industry.

The three positions to disappear will be

the directorate heads for marine and atmospheric sciences, for Earth sciences and for terrestrial and freshwater sciences. Each of these currently has line responsibility for overseeing a range of NERC laboratories and research institutes.

The occupant of the new post will be responsible for the implementation of NERC's scientific strategy for the whole environment. To complement this, the institutes will be formed into two groupings, each intended to enhance their roles as 'contractors' to the council.

Each centre will have its own director, who will be responsible for the professional management and organization of the component institutes (although the directors will be at a lower civil service grade than the previous directorate heads — and the new science and technology director).

One grouping will be a new Centre for Coastal and Marine Sciences, which will include for example both the Plymouth and Dunstaffnage Marine Laboratories. The second is a Centre for Ecology and Hydrology, grouping four separate institutes working in terrestrial and freshwater areas.

The move is partly designed to level the playing field for research groups competing for grants in the Earth, marine and environmental sciences, placing scientists working inside and outside the council on a more equal footing when it comes to applying for research funding.

But it is also intended to pick up on the theme of the white paper by increasing the potential influence of the 'users' of NERC research in deciding which fields should receive priority funding. Indeed, an important part of the new director's responsibility will be to develop NERC's role as a 'customer' for the research that it pays for in both universities and its own institutes.

A senior scientist will be appointed to

take responsibility for what is described as 'technology interaction', and the new director for science and technology will be required to balance the scientific priorities of the academic community with the views of potential users expressed, for example, by the results of the government's technology foresight exercise.

"This is quite a radical change for the NERC," says John Krebs, professor of zoology at the University of Oxford, who took over the new position of chief executive officer of the NERC last month. He says that the reorganization is an attempt to move away from the previous situation which had tended to lock the NERC into committing funds to its institutes rather than universities. "Buying the best science where you can get it is something that all the research councils are moving towards," he says.

The reorganization has been actively encouraged by the Office of Science and Technology which, in line with the government's wish to stimulate a more market-oriented approach to the management of publicly funded research, is keen to see a greater separation between the roles of customers and contractors within the research councils.

But it has also opened up the question of how far the customer/contractor relationship, which in its original formulation by Lord Rothschild was intended to apply to links between research councils and government departments, can legitimately be applied to the workings of the research councils themselves.

Of particular concern to some scientists is the extent to which the council will consider that it still has a long-term responsibility for the scientific health of its individual institutes. "This is certainly an issue which has yet to be resolved," said one observer last week.

David Dickson

Wellcome Trust warns of research 'in peril'

London. The director of the Wellcome Trust, Britain's largest medical research charity, which last year awarded research grants totalling almost £150 million (US\$225 million), has warned that government reforms of the National Health Service (NHS) are placing the future of clinical research in the United Kingdom "undoubtedly in peril".

The warning comes in the latest annual report of the trust, which has recently been forced to reconsider its investment in new laboratory facilities at Hammersmith Hospital in London following the government's refusal to cover the costs of protecting research investments in a merger with the nearby Charing Cross Hospital (see *Nature* 369, 92; 1994).

Bridget Ogilvie, the director of Wellcome, says that the implementation of a 'purchaser/provider' split — with hospital managers seeking to provide services at the lowest possible price — is having "serious consequences" for clinical research. In particular, she writes, the changes are making "enormous demands" on senior medical staff with responsibility for both clinical training and research.

She also says that the trust is watching "with concern" how changes in the funding of research, following the publication of last year's white paper, are being put into practice — and in particular the consequences of the new remit of the research councils to respond to the needs of users of research.

Ogilvie points out that senior government officials have recently condemned efforts by others to subject key members of the community to the Maoist philosophy of continuous revolution. "It is greatly to be hoped that this comment will be implemented by those with responsibility for the university sector, and also the NHS," she writes. □

\$60 million gift for neurobiology research

Cambridge, Massachusetts. **The Harvard Medical School plans to use the investment income from a \$60-million gift — the largest it has ever received — to conduct neurobiological research aimed at resolving some of the mysteries surrounding cerebral palsy.**

The research will concentrate on how nerve cells develop, how they adapt to external stimuli, and how they die, emphasizing the way in which these activities affect and are affected by the condition of cerebral palsy. The donation has been made by Isabelle and Leonard Goldenson in recognition of the support given by scientists at the medical school in the 1950s to their daughter, who suffered from cerebral palsy.

Mr Goldenson is the founder of the American Broadcasting Companies, Inc. and chairman of the executive committee of Capital Cities/ABC, Inc. The school plans to dedicate a newly renovated research building to the Goldensons in September. Although no money from the gift will be used for the renovation, the donation will finance research to be conducted in the new laboratories.

In 1953, the late Sidney Farber of Harvard helped Mrs Goldenson to found the United Cerebral Palsy Research and Education Foundation. This combined the efforts of US medical schools, and members of both medical and technology departments, to help scientists to learn more about cerebral palsy.

Victoria Griffith

Listening to Prozac

SIR — *Listening to Prozac* by Peter D. Kramer is certainly a very bad book, but it deserved a better informed review than that from Charles Medawar (*Nature* 368, 369; 1994). It is impossible to deal here with his entire shoal of red herrings, but several points need to be made.

Prozac is one of five drugs (selective serotonin re-uptake inhibitors, or SSRIs) with the same basic action that are in widespread clinical use; its only major difference from the others is that its effects last longer. This may be advantageous or not for different people, but makes little practical difference in most cases. If Prozac were 'addictive', the same would have to be true of the other SSRIs, but no-one has suggested that this is so.

During the past 35 years, there has in fact been no evidence that any antidepressants — whatever their structure — cause 'addiction' or 'dependence'. Medawar says there is "profound confusion" over the meaning of these terms and, if so, he has certainly added to it. Diabetics are 'dependent' on insulin and people with high blood pressure are dependent on hypotensives, in the sense that they will become ill again if they stop taking the drugs. Many sufferers from depression are in the same position, but this is totally different from the experience of people who take heroin or cocaine as euphorians. They suffer from dose escalation and distress on stopping, not a return to a morbid state that existed before the drugs were first taken.

There is a fundamental difference between drugs that have a euphoriant effect for practically everyone (alcohol, heroin and so on) and those that improve the mood only of people suffering from a mood disorder. Whereas the first category acts almost immediately, the beneficial effect of antidepressants does not begin for about two weeks. People who are not depressed and who take antidepressants only experience side effects — a poor recommendation for a 'recreational' drug. Further, there is no evidence that Prozac (or any other antidepressant) affects personality, as opposed to disordered mood. Kramer's few anecdotal cases certainly provide no such evidence.

The usual dosage of Prozac has been well established for at least ten years at 20 mg, although a range of doses is nothing unusual in pharmacology and there have been recent reports that some patients respond well to less than 20 mg. Every drug has some unwanted side effects, but the safety profile of Prozac is advantageous. Any drug can be misused, but this is no reason to deprive people of its benefits.

Effective drugs that have been shown to have no potential for addiction exist for

the treatment of anxiety — buspirone and the monoamine-oxidase inhibitors. Far from antidepressants being excessively used, all the available evidence shows that many more people could be relieved of unnecessary suffering by the more effective use of these drugs. It would be regrettable if serious depressive illness, often involving the risk of suicide, remained untreated through people being misinformed about the well-established properties of antidepressants.

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SIR — Medawar is largely right in his criticisms of *Listening to Prozac*; in claiming that Prozac — and other antidepressants — is potentially addictive, he is not only wrong, but doubly so.

First he has confused antidepressants and antipsychotic drugs with two other classes of drug: sedatives and stimulants, such as Valium and cocaine. Not only have the former drugs been around for longer than the benzodiazepines, but any clinician knows of the difficulties of getting unwell patients to stay on them, to say little of well ones — hardly suggestive of addictive potential.

The fact that the media claim that many hundreds of people are prescribed Prozac signifies little more than the exaggeration of an anecdote for a good story. Any patient who is put unnecessarily on Prozac will invariably discontinue it within a few weeks, either out of boredom or owing to side effects (and yes, they do occur, even with Prozac). Just ask any psychiatrist in clinical practice.

What makes a drug addictive is the production of feelings of elation, euphoria or ease; growing tolerance and loss of effects over time; and withdrawal symptoms when the drug is stopped. Although antidepressants may have a few mild 'rebound' symptoms on discontinuation, this cannot be equated with dependence. Furthermore, the reason they are sometimes slowly discontinued ('phased withdrawal') is to minimize the likelihood of convulsions, an occasional but unrelated issue.

In denying the beneficial effects of a new and easily tolerable antidepressant, Medawar seems preoccupied with the likelihood of addiction. What then does he have to say about the fact that even tap water can be addictive (psychogenic polydipsia)? The real axe he has to grind is an anti-psychiatry and anti-doctor one; he should at least have the grace to come out and say so.

Robert Kaplan

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SIR — Kramer is clearly fascinated scientifically by the effects of psychoactive drugs on personality, and deeply concerned about the moral and ethical dimensions of what one might call 'personality engineering'. So I am mystified why Medawar calls him "alarmingly disingenuous" for raising these issues publicly and accuses him of seeking to "stimulate mass demand" for the drug.

Medawar also criticizes Kramer for using too many anecdotes about his patients. As observation of patients would seem to be indispensable for a clinician trying to evaluate a drug's effect, I am hard-pressed to understand how one could write about the effects of Prozac without using anecdotes. Does Medawar accept only statements about mortality rates?

Medawar reasonably addresses the issue of whether Prozac may be addictive. I doubt whether he would deny insulin to a patient condemned to diabetes, but would he deny antidepressants to people suffering from another genetically linked disease, chronic depression? His tone — "patients don't crave so long as doctors readily prescribe" — gives the impression that he considers prescription of psychoactive drugs to be the lazy doctor's approach, and ingestion of the drug a moral failing on the part of the patient.

I have for several years been in frequent (often daily) contact with someone who takes Prozac under the close supervision of a psychiatrist. This person, whose family history is rife with chronic depression, has achieved remarkable stability after a grim period of contemplating suicide. There seem to be no adverse side effects. I hope Medawar will pause to consider such anecdotes before trotting out his next denunciation.

Joseph Walder

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Oh Canada!

SIR — The fact that John Maddox was seduced by "majestic" Montreal in February (*Nature* 368, 389–394; 1994) is understandable to this native. Less understandable is his seduction by the separatist Parti Québécois' rationales for secession of Quebec from Canada: Québécois "feel differently in themselves when talking French" and "they want to live more intimately with people like themselves". Bah! This linguistic mawkishness conceals a hard-core jingoism that treats constitutional rights in the Canadian Charter as mere inconvenience. To see behind the front, read Mordecai Richler's *Oh Canada! Oh Quebec! Requiem for a Divided Country* (Penguin, 1992).

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Pandas, people and policy

Stephen J. O'Brien, Pan Wenshi & Lu Zhi

Panda conservation in China has been plagued by controversy and cultural and political differences. But international cooperation, together with new studies identifying the main threats, offer renewed hope for the species' survival.

GIANT pandas (*Ailuropoda melanoleuca*) pose a unique challenge to conservationists, for they are confined in the wild to China, a nation with a tortuous recent history, a massive human population explosion and a cultural and political distinctiveness from Western societies. A combined resolve by Chinese and Western conservationists to stabilize remaining panda populations has blossomed, stumbled and evolved since 1979 when the World Wide Fund for Nature (WWF, formerly the World Wildlife Fund) joined the Chinese Ministry of Forestry to study and preserve the species¹. Since 1987, however, there have been definite advances in giant panda conservation. Here we summarize these developments while making some further recommendations.

The status of wild giant pandas certainly seems grim. There is a total of about 1,100 free-ranging pandas on the basis of a 1985–87 census^{2–4}. Panda ranges have been halved over the past 20 years, the species' present habitat consisting of six unconnected areas of alpine bamboo forest (see map). Continuing human activity, particularly poaching and commercial timber harvesting, would guarantee a steady decline in panda numbers.

Today's pandas are further subdivided into about 24 small populations separated by mountain ranges, rivers, roads, forest clearings and human settlements. Some populations have fewer than 20 pandas, making them demographically^{5,6} and genetically^{1,7–10} vulnerable.

A back-up captive breeding programme has not yet become fully self-sustaining, being continuously supplemented by 'rescued' wild pandas (93 captures in the past 15 years). Between 1936 and 1988, 345 pandas held in captivity produced 67 litters of 106 cubs. Only 32 of these survived more than a year. This infant mortality rate is among the highest for any zoo-bred species^{8–9}.

The frustrations of panda conservation in China have been emphasized by George Schaller, who carried out a ground-breaking WWF-backed study of pandas at Wolong in the early 1980s (ref. 11). *The Last Panda*⁴, a provocative and highly personal account of his experiences, was published last year. *Newsweek*

magazine reported¹² that "Schaller lets loose a shattering attack on the sham called panda conservation. He charges that stupidity, greed and indifference are hastening loss of wildlife." Schaller takes issue both with his sponsors, "two bureaucratic institutions: the Chinese government, with its vast hierarchical structure from the highest level in Beijing to the lowest in our camp; and WWF, far removed in Switzerland from pandas, yet determined to control the local situation", and with Western zoos, driven by greed to display pairs of pandas regardless of the conservation consequences. Schaller's

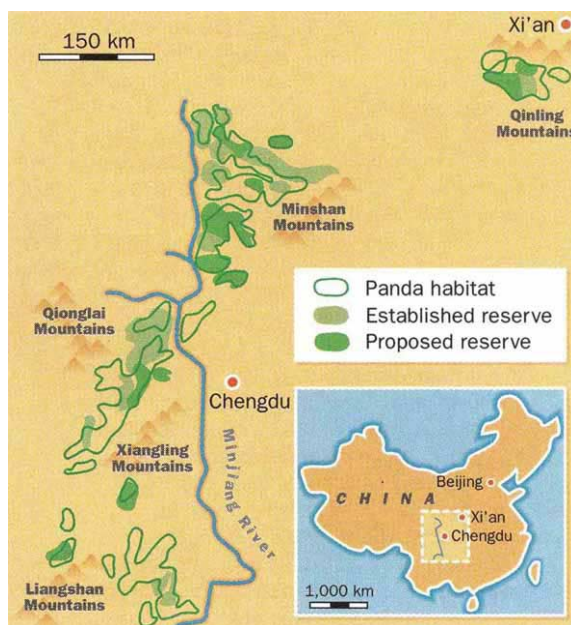
tion we are often asked by local farmers as well as college graduates and government officials. Lack of understanding and ridicule have plagued conservationists of all nationalities in China, particularly in the early 1980s.

But times have changed. In the past dozen years, there have been real advances in giant panda conservation as a result of international cooperation among Chinese ministries, Western experts and environmentalists. When we visited Wolong¹ in 1986, scientists from the WWF and the Chinese Ministry of Forestry were developing a National Conservation Management Plan for the Giant Panda and its Habitat, which was submitted to the Chinese government in August 1989 (ref. 2). The plan proposed the maintenance and expansion of 13 previously established giant panda reserves and the designation of 14 new panda reserves (total area 10,072 km² including 6,500 km² of occupied panda habitat). It would cover almost all of the currently occupied panda habitat and protect 95 per cent of free-ranging pandas. "No human activity" should interfere with the pandas' survival in the reserves. The plan also covers protection of migration corridors between isolated populations.

The WWF's initial estimate of the cost of the plan was RMB90,000,000 (US\$19,000,000). The budget was primarily meant to pay for new reserves, to move out timber companies (including some 10,000 workers) and to resettle

peasants. Relocation would be voluntary and come with a financial incentive. In April 1993, the Ministry of Forestry returned an amended version of the plan. The biggest changes were the cost, which had tripled owing to inflation and additional relocation; the proposed introduction of conservation monitoring stations; and an extension of the implementation period from five to ten years. The Chinese government has allocated a budget of \$13,000,000 and asked that the balance, \$64,000,000, be secured from outside sources. This is a major commitment to giant panda conservation by the Chinese government, but securing the balance remains an obstacle.

Meanwhile, our ecological study of the giant pandas in the Qinling mountains had



Distribution of giant pandas in west central China.

pleas for anti-poaching patrols were ignored^{3,4}. In witnessing Chinese workers' mistakes and indifference in medical procedures, he became sceptical of the value of the technologies for pandas.

It is important to remember that when Schaller made his observations, the Chinese people, in the wake of the Cultural Revolution, were still experiencing considerable social and political turmoil. In China, wildlife has been traditionally considered as a harvestable resource for the benefit of its more than 1 billion people. When the Chinese government introduced laws protecting animals and instituted penalties for violation, yesterday's hunters became today's poacher-criminals. Many people were initially confused: "Why protect pandas?" is a ques-

revealed some encouraging findings¹³⁻¹⁶. The panda population in this isolated 1,500-km² timber reserve has remained stable over 16 years of observation, with estimates of 237 pandas in 1976, 240 in the mid-1980s and 240 in 1992. The age-structure of a study group of 53 pandas in 1993 was optimal. Of 17 radio-collared pandas monitored between 1987 and 1993, we recorded ten births (including at least one twin) and only four deaths. This expansion continues at the edge of both farm settlements and an advancing timber industry that has reduced the available habitat by 20 per cent since 1976.

Things may have improved at Wolong as well. A crew of 40 WWF-trained anti-poaching rangers has been recruited to patrol the habitat, uncover snares and arrest poachers^{1,2,11,14,16}. The WWF also appointed a full-time veterinary researcher in 1991 to assist with panda reproduction¹⁷, disease monitoring¹⁸ and genetic analysis. Natural reproduction among captive pandas at Wolong Research Centre has since improved dramatically, and a preliminary description of endemic carnivore pathogens and paternity analysis are under way. Regular bimonthly surveys of panda traces continue to monitor the wild population.

Cooperation between Chinese zoos and Ministry of Forestry breeding stations has also led to important advances towards a self-sustaining captive population^{16,19}. Between 1989 and 1993, 39 cubs were born and 25 of these survive today. In 1992, a boom year, 13 cubs were born of which 11 survive. In 1993, there were 117 pandas in captivity, 75 of them caught in the wild. And in 1992, a complete international studbook¹⁹ of giant pandas, listing births, deaths, capture locations and other valuable data, was published.

As to the main threats to panda survival, lumbering has ceased in the old reserves, although it continues in the new reserves despite nearly unanimous agreement to implement the management plan. Poaching remains a great problem in some reserves and a potential menace in others; until effective anti-poaching protocols are established, the loss of pandas, driven by a lucrative pelt trade, will continue. Finally, there are the demographic and genetic problems associated with small isolated populations^{1,5,6,16,20}. (The idea that bamboo flowering is a principal threat has been put to rest: pandas migrate, switch bamboo species or simply change elevation to secure nutrients¹⁻⁴.)

The way forward seems clearer than ever. The management plan provides a blueprint for panda conservation, and it should be implemented quickly. In the short term, the timber harvest in all reserves must be stopped immediately. A formal recommendation that timber harvesting in designated reserves should be uncoupled from funding of the plan was

made to Chinese Premier Li Peng by 29 participants at the International Giant Panda Conservation Conference held in Chengdu in September 1993. A month later, a courageous positive response came from Vice-Premier Zhu Rongji. Logging was suspended in Qinling and \$3 million was budgeted for relocation of the 2,300 timber workers; the first \$1 million has already been delivered. This is good news for pandas, but it should be extended to all new reserves, making provision of resettlement financing even more urgent.

There should also be a strict moratorium on 'rescues' of 'abandoned' panda cubs from natural populations. The captive-breeding programme is beginning to do well and should not need more animals. Further, it is now known that mother pandas often 'abandon' their cubs for up to 52 hours for feeding (or even in response to human intrusion)^{13,15}.

But what about funding? A worldwide campaign for support may be part of the solution. Giant pandas are superstars that can bring enormous profits to zoos. Between 1984 and 1991, Western zoos paid hundreds of thousands of dollars for three-month loans of pandas. But such short-term loans have an adverse effect on panda reproduction^{4,21,22}. In 1991, after legal suits, counter suits and slanderous accusations, China announced that short-term loans would be suspended.

Chinese and Western observers have recently proposed a way in which long-term captive panda loans might in fact meet the cost of the management plan. Long-term breeding loans are common in the management of other endangered species²³ and could be introduced with appropriate fees. In May 1993, Adventure World in Japan negotiated for a pair of pandas from Beijing Zoo at a cost of \$10 million for a ten-year breeding loan. Two US centres (San Diego Zoo and Busch Gardens) are working towards a similar deal at a comparable cost. At this rate, six loans would provide the sum required to fund the whole management plan and probably would contribute to a self-sustaining captive population without resort to further removal from the wild. A critical aspect of this recommendation is stringent international monitoring (perhaps by the International Union for the Conservation of Nature, another respected conservation organization or an *ad hoc* committee of Chinese and Western conservation experts) of a 'one-time only' breeding loan to avoid any private deals and to ensure that funds are specially designated for the implementation of the management plan.

With such loans, however, the possibility for political use of giant pandas is real. Giant pandas have featured in international diplomacy in the past (for example, in 1972 when the United States reestablished diplomatic relations with China).

But endangered species should never be held 'hostage' by politics^{26,27}, and a successful panda conservation policy must be totally separated from political issues. The US Department of Interior (US Fish and Wildlife Service) should recognize the overall benefit of long-term loans and put aside complaints about supposedly ulterior motives of zoo officials or imagined threats to wild populations.

No-one likes putting a price on endangered species. Yet these are difficult times, where compromise is productive and conservation has a cost. The solution recommended here may not even be necessary if alternative fund raising and budget plans are successful. Nevertheless, international governing bodies are in place to oversee both the captive breeding and conservation initiatives. The WWF-Chinese plan is a sensible solution, and new data from field ecology to molecular genetics will continue to provide insight and direction for its management. In his final statement on the plan, Fan Zhiyong, a Ministry of Forestry spokesman, pleaded: "Let us save our giant panda together". We may just do that. □

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- O'Brien, S. J. & Knight, J. A. *Nature* **325**, 758 (1987).
- National Conservation Management Plan for Giant Pandas and its Habitat (WWF/Ministry of Forestry of the People's Republic of China, 1989).
- Johnson, K. G., Schaller, G. B. & Hu, J. *Natn. geog. Res.* **4**, 161-177 (1988).
- Schaller, G. B. *The Last Panda* (University of Chicago Press, Chicago, 1993).
- Land, R. *Science* **241**, 1455-1460 (1988).
- Roelke, M. E., Martenson, J. S. & O'Brien, S. J. *Curr. Biol.* **3**, 340-350 (1993).
- O'Brien, S. J. & Evermann, J. F. *Trends Ecol. Evol.* **3**, 254-259 (1988).
- Ralls, K., Brugger, K. & Ballon, J. *Science* **206**, 1101-1103 (1979).
- O'Brien, S. J. *et al. Science* **227**, 1428-1434 (1985).
- Black, F. L. *Science* **158**, 1739-1740 (1992).
- Schaller, G. B., Hu, J., Pan, W. & Zhu, J. *The Giant Pandas of Wolong* (University of Chicago Press, Chicago, 1985).
- Beagley, S. *Newsweek* **121**, 50-56, 12 April 1993.
- Pan, W. *et al. The Giant Panda's Natural Refuge in the Qinling Mountains* (Beijing Univ. Press, 1988).
- Hu, J., Wei, F., Yuan, C. & Wu, Y. (eds) *Research and Progress in Biology of the Giant Panda* (Sichuan Pub. House of Science and Technology, Cheng Du, 1990).
- Lu, Z. *The Population Dynamics, Movements and Social Organization of the Qinling Panda* (PhD thesis, Peking Univ. Beijing, 1992).
- Proc. int. Giant Panda Conserv. symp* (September 1993, Chengdu, Sichuan, China, in the press).
- Chen, M., Zhang, G. & Mainka, S. A. *Zoo Biol.* (in the press).
- Mainka, S. A., Qiu, X. & He, T. J. *Wild. Dis.* (in the press).
- Zhao, Q. *et al. Giant Panda Studbook* (China Assoc. Zool. Garden, Beijing, 1993).
- O'Brien, S. J. *et al. Science* **223**, 1127-1129 (1984).
- Roberts, L. *Science* **241**, 529-531 (1988).
- Roberts, L. *Science* **241**, 1594 (1988).
- Foose, T. J. in *Tigers of the World* (ed. Tilson, R. L. & Seal, U.S.) 304-315 (Noyes, Park Ridge, New Jersey, 1987).
- Soule, M. E. *Cons. Biol.* **1**, 173-174 (1987).
- Diamond, J. & Soule, M. E. *Cons. Biol.* **2**, 121 (1988).

ACKNOWLEDGEMENTS. We are grateful to Z. Fan, C. Hails, D. Kleiman, S. Mainka, J. Martenson, G. Schaller and D. Wildt for discussion.

Directed motion from random noise

Fears that biochemical ratchets as explanations of muscle contraction (among other things) would contradict the Second Law of Thermodynamics have been stilled by an interesting counter-example.

No apologies are required for returning so soon to the question of whether the contraction of muscle fibres, or the transport of macromolecules along microtubules, entails some kind of ratchet mechanism in which molecules are able to move relative to each other in one direction, but are unable to slip backwards (see *Nature* **368**, 287; 1994). For if these processes do involve a biochemical ratchet, that would be important. But there is one snag: in, for example, transport along microtubules, there is no known gradient of chemical concentration or of temperature to determine the direction of the movement. That is another way of saying that the ratchet explanation appears to contradict the Second Law of Thermodynamics, which would be a serious matter.

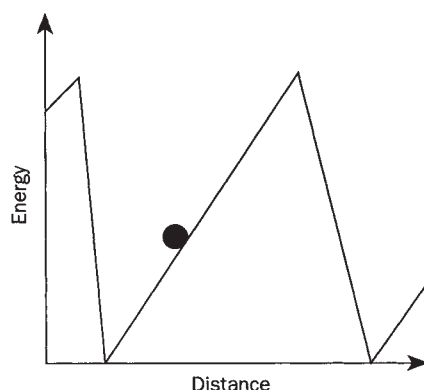
The point can be illustrated with the help of Maxwell's demon. Suppose that microtubules have a periodic structure extending along their length, and that materials being transported along them are normally bound at sites that are also distributed periodically. If the molecule is to move, it must be given at least enough energy to surmount the energy peak between two minima. But what will ensure that it can move only in one direction? A Maxwellian demon at each binding site instructed to prevent backwards movement could do the trick, but that is merely to confess that the Second Law, which outlaws Maxwell's demon, is violated. Is there a way round the difficulty?

The point is of some historical interest because a model of a mechanical ratchet was used in *The Feynman Lectures in Physics* to show that useful work cannot be extracted from thermally driven fluctuations, or 'white noise'. It is simplest to begin with a strictly mechanical one-dimensional ratchet in which an object (the black sphere in the diagram) can move in one direction only, but in a periodic structure with a periodic potential (of which one sawtooth is displayed). Feynman's question (answered in the negative) was whether the asymmetry of the ratchet structure would determine the movement of the particle in one direction rather than the other under random impacts from, say, the molecules of a perfect gas at some non-zero temperature.

It is not difficult to reach the conclusion that there can be no preferred direction of motion. What matters is whether the particle will acquire enough kinetic energy to surmount the sawtooth, and that is as likely to happen in one direction as the other.

But what if the noise is not white noise?

That is the question now taken up by Charles R. Doering, Werner Horsthemke and Jason Riordan (the first and third from Clarkson University, Potsdam, New York, and the second from the Southern Methodist University in Dallas, Texas). Their argument appears in the current issue of *Physical Review Letters* (**72**, 2984–2987; 1994). To simulate the properties of biological structures, they suppose that the motion of the particle in the sawtooth ratchet potential will be over-damped, as in a viscous me-



dium, implying that its motion will be determined by equating its velocity to the sum of all the forces to which it is subjected — the potential gradient and the noise. Feynman's conclusion (that white noise yields no net motion) still applies.

Non-white noise must be defined. In a perfect gas, the motion of different molecules is strictly independent. The chance that an object which is macroscopic (relative to the gas molecules) will acquire enough energy to surmount the potential peak is entirely a matter of chance — the chance that successive impacts will give it enough energy. And these impacts are entirely uncorrelated. An impulse in one direction is just as likely as not to be followed by a second impulse in the same or the opposite direction. One way of constructing non-white noise is to suppose that, instead, there is a time-correlation between successive impulses.

At this stage, it may be remarked that fiddling with the time-correlation offers a ready but trivial way of solving the microtubule problem: simply postulate that all the external impulses are in the same direction, and the particle in the sawtooth will move only in that direction. But that, of course, is simply a way of concealing the interesting question, which is whether it is possible to extract useful work from im-

pulses due to noise that are symmetrical in their direction and on the average zero.

The authors discuss the general case in formal terms, but there is no general solution. Their real interest is to specify non-white noise that can be handled algebraically. The case that proves amenable to treatment is that in which the noise exerts either a positive or a negative force on the particle in the sawtooth, and switches from one state to the other at random times determined by a decreasing exponential function. In other words, the force due to the non-white noise is either in one direction or the other, and each state is converted into the other as if by radioactive decay.

Given that the two different states have the same half-lives, the time-average of the forces due to noise is evidently zero, while the forces are symmetrical. The only asymmetry in the problem is the shape of the sawtooth potential in which the particle is trapped. But the calculations show that there is a net movement of particles in one direction along the periodic sawtooth. Interestingly, the direction is that away from the steepest sawtooth edge. Not surprisingly, the magnitude of the non-white force must exceed a certain minimum before there will be any movement. It may be important that the maximum rate of transport is not that when the non-white forces are very large, but at some intermediate value related to the geometry of the sawtooth.

But what has this to do with the real world? That is what muscle physiologists and others will be asking. But this one illustration that strictly symmetrical non-white noise can provoke asymmetrical movement along an asymmetrical sawtooth may be more relevant than it seems.

All these biomechanical processes appear to be driven by the energy of the hydrolysis of ATP at catalytic sites carried by the molecules involved, which are presumably the source of the non-white noise forces. On the face of things, there is no reason to expect that to be a process favouring one direction of movement rather than the opposite. On the other hand, there is every reason why the non-white forces should be correlated in time; the hydrolysis of consecutive ATP molecules must be limited at least by the time they take to diffuse to the catalytic sites, for example.

Even so, the authors are commendably modest in their claims. "Whether or not nature takes advantage of this kind of effect at the subcellular level," they say, "remains to be seen."

John Maddox

Chance for snowballs in hell

David A. Paige

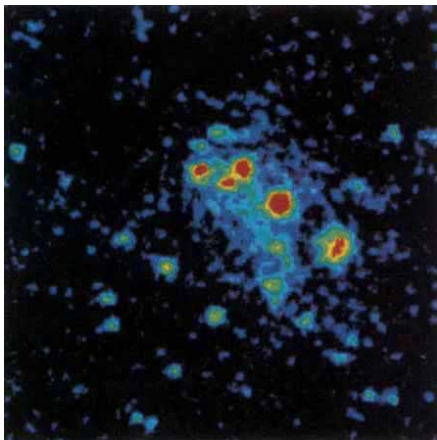
IN October 1991, when radar astronomers^{1,2} first announced that the anomalous reflections they observed at the poles of the planet Mercury were due to the presence of water ice deposits, the news was greeted with a fair amount of scepticism³. However, new higher-resolution radar maps reported on page 213 of this issue⁴ leave little room for doubt: they show that the regions of anomalous reflectivity are localized within small areas whose positions can be made to coincide exactly with the positions of high-latitude impact craters. According to theory, the permanently shadowed floors of these craters are the only places on Mercury that might be cold enough to trap water ice for billions of years^{5,6}.

How can water ice be stable on the closest planet to the Sun? Surfaces on Mercury that are exposed to the Sun's direct rays become very hot, reaching temperatures as high as 700 K at the equator when the planet is closest to the Sun. However, because Mercury's spin axis is almost exactly perpendicular to its orbital plane, the situation at Mercury's poles is very different. To an observer at one of the poles, the Sun never rises or sets, but remains perched at the horizon throughout the year.

Thermal models show that if the surface of Mercury had no topographic relief, then the glancing solar rays that reach the poles would still be strong enough to result in maximum temperatures of about 170 K — cold by terrestrial standards, but still not cold enough to prevent the rapid evaporation of any water ice deposit⁶. However, images obtained by the Mariner 10 spacecraft as it flew by the planet in 1974 and 1975 show that the surface is far from flat; it is pockmarked with impact craters. At high latitudes, crater rims provide shade for the walls and floors within, creating regions that never receive any direct sunlight. The temperatures of these permanently shadowed regions are determined primarily by the fluxes of scattered solar radiation and emitted infrared radiation from the crater walls. Within larger craters, the annual maximum surface temperatures within these permanently shadowed regions should routinely be as low as 100 K (refs 5, 6). At these temperatures, the vapour pressure of water ice is so low that once established, ice deposits should be stable for billions of years — just as they are on the surfaces of icy satellites in the outer Solar System.

Two pieces of good fortune have allowed the Jet Propulsion Laboratory's Goldstone antenna with the Very Large Array¹, and the Arecibo facility in Puerto Rico² where the new results were also

obtained⁴, to reveal the presence of ice in these dark, hidden regions. The plane of Mercury's orbit about the Sun is inclined by seven degrees relative to that of the Earth, which makes it possible for Earth-based radars to 'see' into the interior regions of these craters despite the fact that they are never directly illuminated. Furthermore ice deposits on Mercury give distinctive, bright, depolarized radar



Radar image of the north polar region of Mercury. Many of the bright spots have been identified with known impact craters. The strong echoes may be backscatter from ice deposits in the crater floors.

echoes which make them stand out in stark contrast to their surroundings. Similar echoes have been observed for ice deposits on Mars and the icy satellites of Jupiter⁷.

Theories to explain the depolarized echoes work only if the ice contains few absorbing contaminants such as rocks and soil, and if it is several times thicker than the radar wavelength⁸. The observations do not rule out the possibility that the ice is covered by a thin layer of soil, which would be largely transparent at radar wavelengths. Aside from these few constraints, little else is known about the physical properties of these deposits.

What can be said about the composition, origin and ages of the material that is cold-trapped at Mercury's poles? The fact that the geographical distribution of the observed radar features agrees so closely with that expected for cold-trapped water ice deposits, combined with water's high cosmic abundance, leaves little doubt that water is one of the main volatile constituents. In fact, from what is known at present, one could easily argue that all available niches for stable water ice on Mercury are full. However, there is a possibility that other, more volatile species are stable within the coldest permanently shadowed regions. From past

considerations of sources for possible ice deposits at the poles of the Moon⁹, the most likely sources for the volatile materials now trapped at Mercury's poles include degassing from the planet's interior, comet and meteorite impacts, and the production of water by the reduction of iron in surface soils by the implantation of hydrogen in the solar wind⁸. As the timescales for the supply and loss of volatile materials from these cold regions are difficult to constrain, it is not yet possible to determine whether Mercury's ice deposits are ancient remnants of the planet's early history, or dynamic, contemporaneous features that are actively being replenished.

The presence of ice at Mercury's poles is an exciting new discovery that will have an impact on future efforts to understand and explore the Solar System. Both NASA and the European Space Agency are currently considering low-cost Mercury flyby and Mercury orbiter missions designed to fill gaps in our present knowledge, including the fact that half the planet's surface has never been imaged by spacecraft. The independent detection of ice, and a better understanding of the processes responsible for its presence, should receive high priority in the planning and design of these Mercury missions.

The new results should also spur efforts to search for ice at the poles of the Moon. Watson, Murray and Brown¹⁰ proposed in 1961 that ice might exist in permanently shadowed craters near the lunar poles, but so far no lunar spacecraft, not even the Clementine orbiter launched earlier this year (and now malfunctioning), has been well equipped to detect it. If ice deposits were found on the Moon, they would be exciting targets for robotic exploration, as well as resources that could improve the chances of long-term human habitation. For present-day space scientists and space explorers, though, the radar results demonstrate something equally valuable. The Solar System still contains many secrets that lie waiting to be discovered — some hidden in places where the Sun never shines. □

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1. Slade, M. A., Butler, B. J. & Muhleman, D. O. *Science* **258**, 635–640 (1992).
2. Harmon, J. K. & Slade, M. A. *Science* **258**, 640–643 (1992).
3. Chapman, C. R. *Nature* **354**, 504–505 (1991).
4. Harmon, J. K. *et al.* *Nature* **369**, 213–215 (1994).
5. Paige, D. A., Wood, S. E. & Vasavada, A. R. *Science* **258**, 643–646 (1992).
6. Ingersoll, A. P., Svitek, T. & Murray, B. C. *Icarus* **100**, 40–47 (1992).
7. Muhleman, D. O., Butler, B. J., Grossman, A. W. & Slade, M. A. *Science* **253**, 1508–1513 (1991).
8. Butler, B. J., Muhleman, D. O. & Slade, M. A. *J. geophys. Res.* **98**, 15003–15023 (1993).
9. Arnold, J. R. *J. geophys. Res.* **84**, 5659–5668 (1979).
10. Watson, K., Murray, B. C. & Brown, H. J. *geophys. Res.* **66**, 3033–3045 (1961).

Matching speed and stability

Robert L. Baldwin

A NEW view of the protein-folding process, based on Monte Carlo simulations, is put forward by Šali, Shakhnovich and Karplus on page 248 of this issue¹ (details of the approach are given elsewhere²). It provides a plausible answer to a long-standing riddle: if the folded structure of a protein is determined by its thermodynamic stability (Anfinsen³), and if the kinetics of conformational search are slow because of the enormous number of possible conformations (Levinthal⁴), then how is it possible for a protein to fold up along a pathway that allows rapid folding and nevertheless arrive at the thermodynamically most stable structure?

Cyrus Levinthal was interested by this question, and in 1987 he suggested a solution (quoted in a report⁵ of a meeting on protein folding): he proposed that nature selects sequences that have the ability both to fold rapidly and to arrive at thermodynamically stable structures. Levinthal was not able to suggest a mechanism by which natural selection would operate to select these sequences, nor could he suggest experiments to test his answer. Now, by simulating the folding process using the Monte Carlo method, Karplus and co-workers^{1,2} find an answer to the riddle that is close to Levinthal's proposal.

They assume that the process by which a protein folds is governed by a potential function expressing the interactions that can occur within the polypeptide chain. (When Levitt and Warshel⁶ proposed in 1975 that the folding pathway of a protein could be predicted by such an approach, they startled the experimentalists and changed their way of thinking about the problem.) They use a potential function of the Miyazawa-Jernigan⁷ type, in which amino-acid residues make pairwise interactions that can be evaluated from their contact frequencies in structures in the protein database, and a highly simplified protein model (a 27-bead self-avoiding chain, that folds by occupying sites on a cubic lattice). The total number of conformations that can be assumed by any sequence is large: after a fast collapse of the unfolded chain to a random, semi-compact globule, there are about 10^{10} possible conformations in the globule. Karplus and colleagues are able to enumerate all possible conformations and to assign an energy to each folded or partly folded form: the native structure is defined as the energetically most stable structure. They investigate the folding kinetics of 200 random sequences and find that a fraction of these are fast folding (30/200); 146 sequences do not fold at all within the number of

Monte Carlo steps allotted for folding.

The fast-folding sequences have a special property, in that there is a wide gap in the energy spectrum between the most stable structure and the next most stable ones. Slow-folding sequences have nearly continuous energy spectra, with the most stable structure being close in energy to nearby structures. Because a fast-folding sequence has a native structure that is stable in conditions where all partly folded forms are unstable, it has no problem in arriving at the most stable structure. So Karplus and co-workers have identified a connecting link between the two parts of the riddle: the ability to fold rapidly is possessed by the same sequences that can form thermodynamically stable structures.

Their finding suggests a mechanism by which nature could select these sequences. Polypeptide chains that remain unfolded in the cell are broken down by specific proteases⁸. Thus, selection of fast-folding sequences should give structures that are thermodynamically stable, if this result holds true for real proteins.

The standard test for thermodynamic stability of a protein structure is whether it unfolds and refolds reversibly without hysteresis. This question has been studied carefully for some small proteins, and they have been shown to satisfy the test rigorously⁹: even if the kinetics of unfolding and refolding are complex, with two or more kinetic phases, the apparent rate constant for each kinetic phase is the same for unfolding as for refolding in the same conditions, inside the unfolding transition zone.

Examples are known of proteins that do not have the ability to unfold and refold reversibly. Members of the serpin family can exist in two different folded conformations, and work on antithrombin indicates that a transition between the two conformations can occur without the intervention of a processing step¹⁰; the biologically active form appears to be the metastable form. So there is the possibility that, in some cases, the native conformation is determined by the folding pathway, rather than by the most stable structure.

In the Monte Carlo simulations, mol-

ecules with a given sequence undergo folding on many different pathways at the same time^{1,2}. The simulations therefore indicate that there is no unique pathway of folding, and no unique transition state. On each of these pathways there is a high-energy intermediate that is close in structure to the native form, and thereby resembles the unique transition state sometimes invoked by experimentalists; however, the folding of a 27-bead chain involves about 10^3 such 'transition states'¹. Until now, experimentalists have drawn an analogy between protein folding and an ordinary chemical reaction that has a defined series of intermediates and a single rate-limiting step. An immediate challenge for them arising from the findings of Karplus and co-workers is to determine whether or not the folding of real proteins has a unique transition state.

A study by Englander and co-workers¹¹ of alternative folding pathways for cytochrome *c* fits in neatly with the new view. They find that a prominent kinetic folding intermediate accumulates because there is a transient barrier to folding, one probably caused by the presence of a non-native haem ligand in the denatured protein. When this barrier is removed, folding can occur on a 'fast track'.

In 1985, Harrison and Durbin¹² argued that experimentalists were thinking incorrectly about protein folding when they compared the process to an ordinary chemical reaction. They proposed that protein folding should instead be compared to the assembly of a jigsaw puzzle, which can start anywhere and proceed along innumerable pathways. Their model predicted that, although there may be folding intermediates, their structures should consist of a random assortment of substructures of the native protein. Thus, the jigsaw puzzle model was discredited when it was found by pulsed hydrogen exchange studies that kinetic folding intermediates have specific secondary structures (for review see ref. 13). In retrospect, it seems that Harrison and Durbin may have had the right idea, although their model was too simple to predict the existence of specific folding intermediates.

One of the conspicuous properties of the Monte Carlo simulations^{1,2} is the presence of well-populated intermediates during the kinetics of folding. Before trying to use the simulations to enquire

1. Šali, A., Shakhnovich, E. I. & Karplus, M. *Nature* **369**, 248–251 (1994).
2. Šali, A., Shakhnovich, E. I. & Karplus, M. *J. molec. Biol.* **235**, 1614–1636 (1994).
3. Anfinsen, C. B. *Science* **181**, 223–230 (1973).
4. Levinthal, C. *J. Chim. Phys.* **65**, 44–45 (1968).
5. Baldwin, R. L. in *Protein Structure, Folding and Design*, Vol. 2 (eds Oxender, D. L. & Fox, C. F.) 313–320 (Liss, New York, 1987).
6. Levitt, M. & Warshel, A. *Nature* **253**, 694–698 (1975).
7. Miyazawa, W. & Jernigan, R. L. *Macromolecules* **18**, 534–552 (1985).
8. Goldberg, A. L. & Rock, K. L. *Nature* **357**, 375–379 (1992).
9. Ikai, A., Fish, W. W. & Tanford, C. *J. molec. Biol.* **73**, 165–184 (1973).
10. Carrell, R. W., Evans, O. & Stein, P. E. *Nature* **353**, 576–578 (1991); **364**, 737 (1993).
11. Sosnick, T. R., Mayne, L., Hiller, R. & Englander, S. W. *Nature struct. Biol.* **1**, 149–156 (1994).
12. Harrison, S. C. & Durbin, R. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4028–4030 (1985).
13. Baldwin, R. L. *Curr. Opin. Struct. Biol.* **3**, 84–91 (1993).
14. Hughson, F. M., Barrick, D. & Baldwin, R. L. *Biochemistry* **30**, 4113–4118 (1991).
15. Matouschek, A. *et al.* *Nature* **346**, 440–445 (1990).
16. Pitts, O. B. in *Protein Folding* 243–300 (ed. Creighton, T. E.) 243–300 (Freeman, New York, 1992).

about essential properties of real folding intermediates, the following problem should be taken into account. In the simulations, the potential function has been chosen to mimic the folding behaviour of native proteins. Each pairwise interaction between two residues, when present, has the same strength in a folding intermediate as in the native protein. But site-directed mutagenesis experiments on folding intermediates^{14,15}, as well as general characterization of equilibrium molten globule forms¹⁶, indicate that side-chain interactions in folding intermediates are loose, weak and relatively nonspecific

compared to those found in close-packed native structures; this is probably because the interacting residues are farther apart in the folding intermediates. If the problem of correctly representing the differences between the interactions in folding intermediates and in native proteins can be solved, and if much longer chains can be analysed, it seems likely that simulations of folding intermediates will provide valuable hints about their true nature. □

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ORIGINS OF LIFE

Chemical replication

James Ferris

ONE of the chief problems with the 'RNA world'^{1,2}, the scheme postulated for the origin of life in which RNA-like molecules catalyse their own replication, is that of getting beyond the complementary copying of an RNA-like template. Orgel

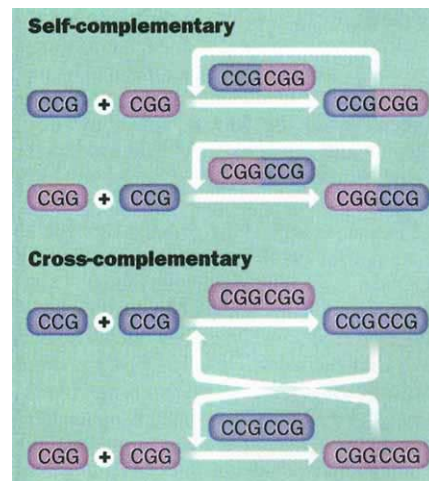
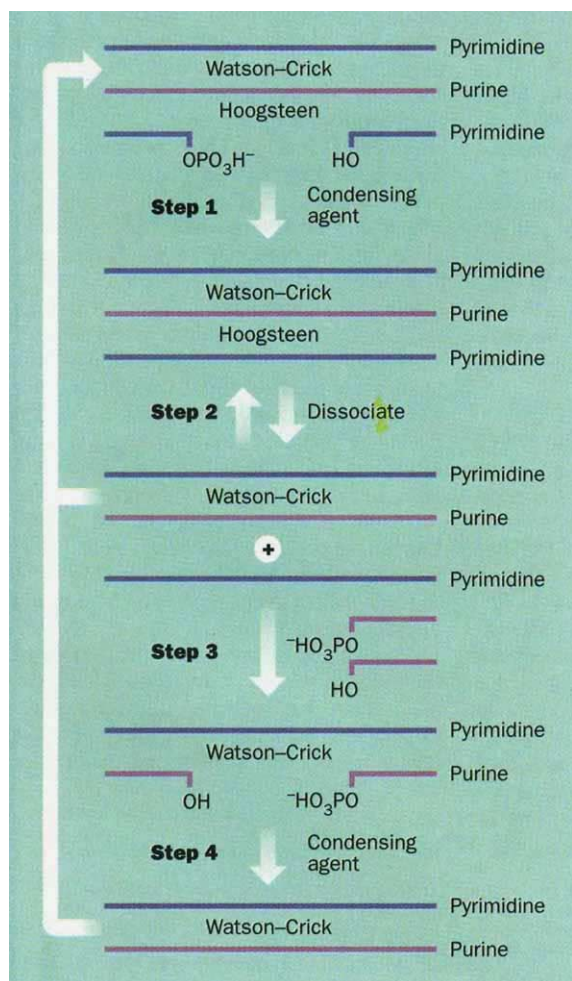
and co-workers³ have demonstrated that activated mononucleotides can be assembled on a pyrimidine-rich oligonucleotide template to form the complementary purine-rich strand, but purine-rich strands cannot then act as templates on which a copy of the original strand can be formed. Two papers^{4,5} in this issue now suggest a way around this problem: they show that small preformed DNA-like oligomers can act as templates for linking still smaller fragments into exact copies of pyrimidine-containing oligomers.

Triple helices are used by Li and Nicolaou⁴ as the replicating intermediates in the synthesis of purine-rich and pyrimidine-rich oligonucleotides. In this approach, which builds on research from the laboratory of Peter Dervan⁶, the starting point is a double helix in which one of the constituent 24-mer chains is composed exclusively of purine nucleotides and the other 24-mer exclusively of pyrimidine nucleotides. This double helix binds two complementary dodecameric pyrimidine oligomers by Hoogsteen (non-Watson-Crick) base pairing between purines and pyrimidines (as shown in the reaction scheme to the left). Addition of a condensing agent results in the ligation of the two dodecamers to give a 24-mer identical to the original pyrimidine oligonucleotide in the double

helix (step 1). As the newly synthesized 24-mer is bound in the triple helix by weaker Hoogsteen base pairs, it is more easily dissociated than the two original oligomers held by Watson-Crick hydrogen bonding (step 2). Next, the dissociated oligomer undergoes Watson-Crick association with two complementary purine dodecamers (step 3), and these, finally, are ligated upon addition of a condensing agent to form more of the starting double helix (step 4).

The net result is the replication of the initial double helix. In principle, the amplification process could result in an eight-fold increase in the amount of the double helical DNA after three cycles; in practice, the increase is five-fold.

Sievers and von Kiedrowski⁵ take a different line of approach. They have investigated the simultaneous replication of four hexameric DNA oligomers by ligation with complementary trimeric oligonucleotides. Two hexamers catalyse the formation of identical self-complementary oligomers and the other two form cross-complementary products as shown schematically below.



As oligomers bind to templates more strongly than do monomers, it is possible in this approach to replicate an oligomer that contains as many purines as pyrimidines, something that was impossible in earlier attempts to construct complementary oligomers from monomers. The rate constants for oligomer synthesis are consistent with an autocatalytic process of reaction order 0.5. This is less than the exponential replication observed in biological systems because the self-association of the hexanucleotide products inhibits the binding of the trimeric starting compounds, but is faster than non-replicating processes.

Do these results establish that the problems of replicating RNA in the absence of catalytic proteins in the proposed RNA world have been solved? A pessimist would note that the oligomers used in these studies are not freely self-replicating

Fair copies — DNA replication via triple helices (here shown untwisted), as used by Li and Nicolaou⁴.

but require the strong intervention of the chemists.

For example, in the work of Sievers and von Kiedrowski, special groupings are used to block reaction on the termini of the hexamers, and the oligomers are coupled together via a phosphoramidate grouping which forms more readily than the corresponding phosphodiester bond found in living systems. In both experiments, the templates and replicating oligomers are carefully chosen and synthesized, the condensing agent is added at the proper time and, in the case of replication via the triple helix, the double-helical product is purified between replication cycles. The chosen oligomers are of DNA rather than the RNA which is thought to have preceded DNA, as DNA is easier to synthesize. And Li and Nicolaou's system uses only palindromic sequences, so that the newly formed strands are identical to the starting pyrimidine oligomer (that is, they have the same 3'- and 5'-terminal groups). In addition, there is the question of the prebiotic pathways to the activated mononucleotides from which the oligomers are constructed.

An optimist, on the other hand, would note that the oligomers required for replication could have been formed by the mineral-catalysed condensation of activated mononucleotides⁷. The sequence information present in these 10- to 20-mers could have been preserved by the above replication processes and the chains elongated by occasional condensation reactions, as Li and Nicolaou suggest. Eventually the oligomers could have evolved in size and catalytic versatility so that they could carry out the synthetic processes required to initiate the RNA world^{8,9}.

My own view is one of cautious optimism. The new work demonstrates that the unsolved problem of information storage by RNA replication is not as intractable as it previously appeared, and we are a small step closer to understanding possible pathways to life. But my guess is that catalysts other than RNA oligomers were involved if replication processes similar to those described here eventually led to the origins of life. □

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1. Gilbert, W. *Nature* **319**, 618 (1986).
2. Joyce, G. F. *Nature* **338**, 217 (1989).
3. Inoue, T. & Orgel, L. E. *Science* **219**, 859-862 (1983).
4. Li, T. & Nicolaou, K. C. *Nature* **369**, 218-221 (1994).
5. Sievers, D. & von Kiedrowski, G. *Nature* **369**, 221-224 (1994).
6. Leubke, K. J. & Dervan, P. B. *J. Am. chem. Soc.* **111**, 8733-8735 (1989).
7. Ferris, J. P. & Ertem, G. *J. Am. chem. Soc.* **115**, 12270-12275 (1993).
8. Bartel, D. P. & Szostak, J. W. *Science* **261**, 1411-1418 (1993).
9. Lehman, N. & Joyce, G. F. *Nature* **361**, 182-185 (1993).

METEORITICS

Mbale impact shower

NINETEEN ninety-two was a good year for meteorite observers. Following the tale of the car-crunching Peekskill meteorite in the United States (D. Hughes *Nature* **367**, 596; 1994, and P. Brown *et al.* *Nature* **367**, 624-626; 1994) comes a detailed report by P. Jenniskens *et al.* of the meteorite that broke up over a heavily populated area of Uganda on 14 August 1992, raining debris for kilometres around (*Meteoritics* **29**, 246-254; 1994). The young boy shown here has the distinction of being the first person known to have survived being hit on

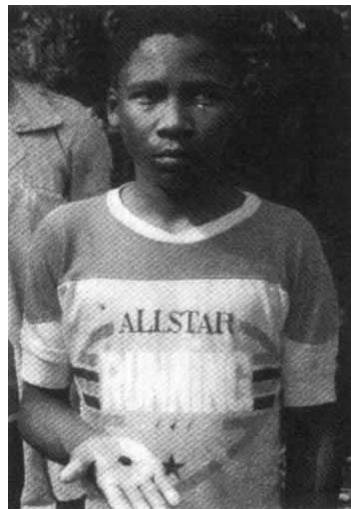
the head by an extraterrestrial. He was lucky: the fragment in his hand weighs a mere 3.6 g, and had the edge taken off its speed by the leaves of a banana plant before it hit him. Had it been one of the larger chunks shown below, he might not (as the researchers put it) have "remained in a position to recover the stone."

Eyewitness accounts of the fall were dramatic. The first intimations came in the form of a rumbling explosion at 3:40 pm local time, at which many industrial workers fled, mistaking it for a bomb blast. For the following few minutes, a "smoke trail" and a compact cloud of dust were visible in the sky, and stones

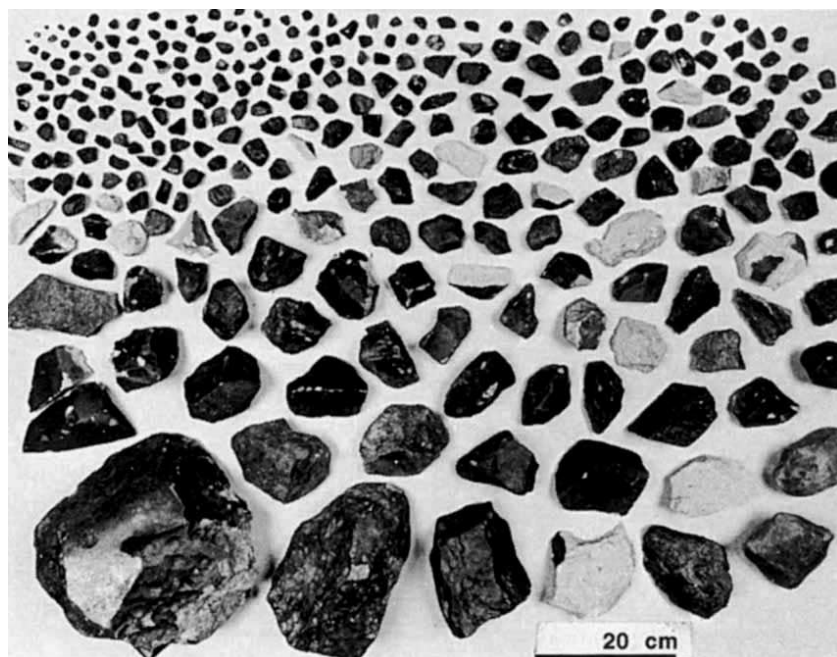
rattled down in and around the city of Mbale accompanied by a sound "like gun fire". The shower caused no casualties and surprisingly little damage: one fragment punched a hole through the roof of the Mbale railway station, another crashed into a cotton factory and a third hit a house roof. Other fragments were less easy to find, but a week-long field expedition shortly after the event located nearly fifty impact sites, and a fine collection of fragments ranging in mass from 0.1 g (top left in the photograph below) to 27 kg (bottom left).

In the months that followed, when they were no longer hampered by the dense vegetation and swamps of the rainy season, members of the local population gathered a further 41 kg of material. Including finds bought from dealers, the tally by October last year was 863 fragments with a total mass of 150 kg. The mass distribution over the strewn field (3×7 km) allows a rough estimate of the original trajectory and orbit, which apparently just reached the asteroid belt. And analyses of short-lived radioactive isotopes, whose production depends on the size of the original meteorite, give an upper mass limit of about 1,000 kg.

Lindsay Matthews



P. Jenniskens/Dutch Meteor Society



National Museum of Natural History, Leiden

Roger W. Sperry (1913–1994)

ROGER Sperry, co-winner of the 1981 Nobel Prize in Physiology or Medicine with Torsten Wiesel and myself, died on 17 April in Pasadena, California, of a heart attack. For many years he had suffered from a neuromuscular degenerative disease, but until recently had continued to be active in thinking and writing about the brain, consciousness and the mind. His contributions to neurobiology were titanic.

I first came across Sperry in the fall of 1953, when I heard him speak at an international physiological congress in Montreal. I had just begun my training in clinical neurology, and had not yet done any research. Sperry's talk came as a revelation.

It is hard today to recapture our state of knowledge of the nervous system in the early 1950s, a time when it was widely thought that the wiring of the brain came about largely through experience. In his talk, Sperry described the simple experiment of surgically interchanging the tendinous insertions of flexor and extensor muscles, or the nerves that supplied them, in a limb of a rat, to see whether the nervous system would relearn to use the muscles properly. The relearning never took place. Even the circuits responsible for spinal reflexes such as limb withdrawal in response to a painful stimulus to the foot remained quite unchanged.

A similar simplicity and lucidity characterized all of Sperry's work on neural development during that era. One of his best known developmental studies established that in a fish the fibres of a severed optic nerve grew back precisely to their former targets in the brain, even if at the time of severing the nerve the eye was rotated 180° in its socket. Here no adaptive relearning or rewiring of the circuits responsible for normal visual behaviour seemed to occur, so that the fish continued to snap downwards at bait placed above it. Such experiments suggested that when individual nerve fibres in a growing nerve trunk find their proper targets they do so by specific chemical cues that somehow recognize complementary cues in the targets. These ideas have still not been proven directly, but they have had a profound influence on the entire experimental field of neurodevelopment, today one of the most active branches of neurobiology.

In the 1950s the nervous system, and in particular the cerebral cortex, was supposed by some (who were taken seriously) to function not by nerve conduction and synapses but by a poorly spelled out process of electrical fields or waves in a volume conductor. Two experiments cleanly disposed of these ideas. In one, Sperry inserted into the cortex many tanta-

lum plates or metal wires, which should have profoundly disturbed or short-circuited any such currents. In the second, he diced up the cortex with radially arranged pieces of insulating mica. In neither experiment was cortical function seriously disturbed. This was before Vernon Mountcastle's discovery of cortical columns, but Sperry did know of the pre-

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Sperry: titanic contributions to neurobiology.

dominance of radial cortical connections from the work of Santiago Ramón y Cajal and Lorente de Nó, and realized that the inserts should leave these connections relatively intact.

My next encounter with Roger Sperry was again indirect; in 1955 I found myself in the army and posted to Walter Reed Army Institute of Research, sharing a suite of laboratory cubicles with Ronald Myers, who had just got his PhD under Sperry at the University of Chicago. Myers's thesis topic involved behavioural studies of deficits resulting from severing the corpus callosum in cats. Up to that time, no one had any idea of the function of this huge bundle of nerve fibres, which connects the two hemispheres; together, Myers and Sperry showed that it had a very specific function in vision, and this was the beginning of the split-brain studies.

In the 1960s, Sperry, along with Joseph Bogen, Michael Gazzaniga and others, extended this line of research to humans. The work appeared first in two beautiful papers in *Brain*, in 1965 and 1967. Almost overnight a wealth of facts and concepts became available. The left hemisphere was known to be largely responsible for speech, but the new observations showed that the right hemisphere has language capabilities too, comprehending much of

what it hears. It could even be shown that certain specific functions are better done by the right hemisphere than the left; for example the left hand (hence the right hemisphere) could carry out some visuospatial tasks far better than the right. In one marvellous passage we find a description of the right hand coming across and messing up what until then had been a successful three-dimensional drawing of a cube by the left hand. In these papers we learned that one person could have, literally at one and the same time, two consciousnesses.

Many of these ideas became distorted when they percolated down to the public, and one could easily get the impression that the right hemisphere was 'for' emotions and art, and that the left was 'for' reasoning and other dry intellectual pursuits. The original papers in *Brain* are the best antidote to such simplifications. They are highly readable and well within the grasp of a high-school student.

In later life, Sperry became increasingly interested in theories of mind and consciousness, concentrating on the relationship between mind and consciousness and ethical values. Many of his fellow neurobiologists could not easily follow his arguments, which seemed to come closer to philosophy than to neurology. But one could sympathize with his contention that brain mechanisms would never be understood solely on a basis of the chemistry and biophysics of single nerve cells. The revolution in these areas in the past generation has made it abundantly clear that without such a basis brain mechanisms are totally out of reach. Sperry's point was that more than chemistry and biophysics is required. It is like the relationship between chemistry of bricks and mortar, and the finished cathedral.

Our week together in Stockholm, in December 1981, with Torsten Wiesel, was marvellous fun. Our family and his were next-door neighbours at the Grand Hotel. In one lovely incident, just before the first banquet, a knock came at our door. It was Roger Sperry's son, holding an untied white bow tie in his hand. "Does anyone have any idea what to do with this?", he asked. I, of course, had no idea, because I have too little sense of style to use anything but the already-tied kind. But our youngest son, who plays the trumpet, had had to wear formal attire so often at concerts that he had become an expert in the difficult procedure. So Paul went next door and tied all the Sperry family's bow ties.

David Hubel

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Courtesy of Caltech

Histidine kinases hog the limelight

David A. Hughes

In bacterial cells, histidine kinases are big players in mediating responses to diverse environmental signals through changes in gene expression and cell behaviour. These kinases were thought to be confined to prokaryotic cells, until late last year when putative histidine kinases were identified in both plants and yeast^{1,2}. In their report on page 242 of this issue³, Maeda *et al.* make the latest contribution to this fledgling field — they show that a yeast histidine kinase regulates the activity of a 'MAP kinase'-like protein kinase cascade, thus bringing together a prototypical prokaryotic signalling module with a eukaryotic one.

In prokaryotic signal transduction, the first part of the so-called 'two-component' system consists of a histidine kinase which autophosphorylates on a histidine residue within its catalytic kinase domain in response to an environmental stimulus. The phosphate is transferred to an aspartate residue on the second component, a response regulator, which then binds to an output protein to effect a cellular response. This basic form of the two-component system has been adapted to suit many different signalling pathways in bacteria⁴. In some systems the histidine kinase is a transmembrane protein that acts as a 'sensor' for a signal; in others, it is a cytoplasmic protein that interacts with a separate transmembrane sensor.

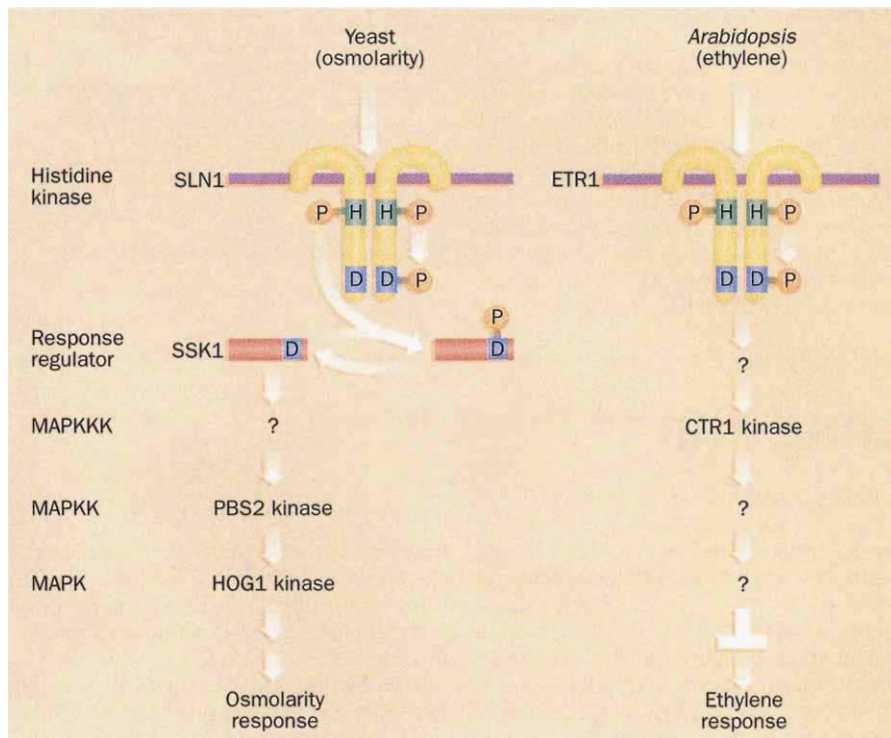
The histidine kinases SLN1 in the yeast *Saccharomyces cerevisiae*² and ETR1 in the flowering plant *Arabidopsis thaliana*¹ illustrate another variation found in some bacterial systems; here, a histidine kinase domain in the amino-terminal region is joined to a response-regulator domain in the carboxy terminus. The presence of hydrophobic sequences resembling membrane-spanning domains suggests that both of these histidine kinases are integral membrane proteins, with an extracellular 'sensor' domain and cytoplasmically located histidine-kinase and response-regulator domains (as depicted in the figure).

What of the function of these eukaryotic histidine kinases? The *Arabidopsis* ETR1 gene was identified by mutations that block the response to ethylene gas⁵, a plant hormone, implying that the ETR1 histidine kinase is a signal transducer for ethylene. Hints as to how this ethylene-response pathway functions in biochemical terms come from the finding that a putative serine/threonine protein kinase, CTR1, functions downstream of ETR1 as a negative regulator of the ethylene response⁶. The yeast SLN1 gene was

identified by mutations that interact with a protein degradation pathway (the 'N-end rule' pathway), but a clue that protein phosphorylation was involved in SLN1 function came from the finding that overexpression of the tyrosine phosphatase PTP2 could suppress an *sln1*-deficient mutant². These studies did not, however,

analyses, the authors present a model in which the SLN1 histidine kinase regulates the phosphorylation state of SSK1: phosphorylated SSK1 is inactive whereas the unphosphorylated form regulates a downstream target.

The most intriguing aspect of this work comes from the analysis of two of the other *sln1* suppressors, which turned out to be mutations in previously described genes: *HOG1*, which encodes a mitogen-activated protein (MAP) kinase homologue⁷, and *PBS2*, which encodes a putative MAP kinase kinase (MAPKK) homologue⁸ that is likely to activate



Two-component systems in eukaryotes. In yeast, changes in osmolarity lead to autophosphorylation on histidine of the SLN1 protein, shown as a homodimer. Phosphotransfer to aspartate residues on SLN1 itself or on the response regulator SSK1 then occurs. Phosphorylation of the aspartate on SLN1 is necessary for its activity whereas phosphorylation of the aspartate on SSK1 inhibits its activity. The active, non-phosphorylated form of SSK1 activates a MAP kinase-like cascade. In *Arabidopsis* the histidine kinase ETR1 may regulate CTR1, a putative MAPKKK, which is required to inhibit the ethylene response. H, histidine-kinase domains; D, response-regulator domains.

identify the immediate downstream targets of the histidine kinases. Following the logic of the bacterial systems, one would predict that phosphate is transferred from CTR1 and SLN1 to another protein carrying a response-regulator domain, even for these histidine kinases with an integral response-regulator domain.

Maeda *et al.*³ now show that the yeast SLN1 histidine kinase does indeed have a separate response-regulator protein, designated SSK1. The *SSK1* gene was identified as one of four genetic complementation groups that could suppress the lethality caused by loss of the SLN1 histidine kinase. From the results of their genetic

HOG1 by phosphorylation. The MAP kinases have been the centre of attention in the past few years, primarily because they have been strongly implicated in the control of proliferation and differentiation in mammalian cells (see ref. 9 for a general discussion). An important lesson that has emerged from genetic studies in yeast is that a single cell can use independent MAP kinase-like pathways to control different cellular responses¹⁰. In *S. cerevisiae* three MAP kinase pathways have been identified, one of which is the HOG1 pathway involved in adaptation to osmotic stress⁷.

The conclusion to be drawn from the analysis of Maeda *et al.* is that the SLN1-

SSK1 two-component system regulates the HOG1 MAP kinase pathway, and this is supported by a biochemical experiment demonstrating tyrosine phosphorylation of HOG1 in response to induction of a constitutively active, unphosphorylated SSK1 protein. The two-component system is not the whole story regarding activation of the HOG1 pathway, however: loss of either HOG1 or PBS2 function confers sensitivity to high osmolarity whereas loss of SSK1 does not, meaning that there are probably several inputs to this pathway. Much remains to be uncovered in this surprising union of signalling pathways, particularly concerning the mechanism of activation of the kinase cascade by SSK1.

It is worth considering a similar (and admittedly speculative) model for the ethylene response in *Arabidopsis* and the role of the putative histidine kinase ETR1 and the serine/threonine kinase CTR1. When *CTR1* was cloned and sequenced it was noted that the predicted product was most similar to the Raf family of protein kinases which are MAP kinase kinase activators (MAPKKs)⁹. Maybe the

plant histidine kinase ETR1 also regulates a MAP kinase-like cascade (see figure)? It would be surprising if two-component systems, perhaps coupled to hitherto unknown MAP kinase-like pathways, were not used by higher organisms, including ourselves, in mediating the cellular response to small molecules. □

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1. Chang, C., Kwok, S. F., Bleecker, A. B. & Meyerowitz, E. M. *Science* **262**, 539–544 (1993).
2. Ota, I. M. & Varshavsky, A. *Science* **262**, 566–569 (1993).
3. Maeda, T., Wurgler-Murphy, S. M. & Saito, H. *Nature* **369**, 242–245 (1994).
4. Bourret, R. B., Borkovich, K. A. & Simon, M. I. A. *Rev. Biochem.* **60**, 401–441 (1991).
5. Bleecker, A. B., Estelle, M. A., Somerville, C. & Kende, H. *Science* **241**, 1086–1089 (1988).
6. Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A. & Ecker, J. R. *Cell* **72**, 427–441 (1993).
7. Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. & Gustin, M. C. *Science* **259**, 1760–1763 (1993).
8. Boguslawski, G. & Polazzi, T. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5848–5852 (1987).
9. Egan, S. E. & Weinberg, R. A. *Nature* **365**, 781–783 (1993).
10. Ammerer, G. *Curr. Opin. Genet. Dev.* **4**, 90–95 (1994).

CHEMICAL PATTERNS

Long-range inhibition

J. Boissonade

OVER forty years ago, Alan Turing pointed out that diffusion need not always act to smooth out inhomogeneities in a chemical system. On the contrary, if a system could be found in which the reactants diffused at different rates, and in which the faster components inhibited reaction whereas the slower components promoted it, stationary patterns might be set up. Long years later, experimental evidence for the arrays of spots predicted by Turing finally emerged^{1,2}, spurring renewed interest in chemical dissipative structures. On page 215 of this issue, researchers at the University of Texas³ describe how they have started to uncover a still larger class of chemical patterns induced by long-range inhibition — in particular, spots that replicate, grow and die in uncanny resemblance to living things.

Competition

Chemical systems kept far from equilibrium by a permanent supply of fresh reactants can show self-organization resulting from competition between reaction and diffusion. The essential feature is that the nonlinear kinetics present two antagonistic processes: an activation process such as autocatalysis and an inhibitory process. Systems that meet these criteria are sometimes known as 'active media'. The same prerequisites are

needed to produce multiple stable states, excitability, periodic or chaotic oscillations and other self-organization phenomena, in systems kept homogeneous by mixing.

The earliest spatial structures seen in excitable or oscillatory chemical media were travelling waves⁴ — target (bullseye) patterns and spiral waves. The role of diffusion here is simply to propagate an excitation — generated by a localized inhomogeneity — throughout the reaction medium. Oscillations can become synchronized or desynchronized.

Following the development of reactor systems in which nonequilibrium conditions can be sustained under constant flow-through of reactants⁵, other types of pattern have been observed. These chemical reactors contain thin ribbon-like or disk-shaped pieces of gel in which the reactants are dispersed. The gel hinders convective motions of the chemical species, and opposite edges are kept in contact with reservoirs of reactants, which diffuse into the gel and replenish those that are consumed.

It was in this type of device that Turing patterns were first seen experimentally at the end of 1989^{1,2}. Unlike wave patterns, Turing structures are stationary and represent a spontaneous instability of the stationary state. They correspond to periodic variations in the concentrations

of the reactants, with a precise wavelength which depends on the reaction rate and diffusion coefficients. The chemical system in which these structures were seen involved the reactants chlorite, iodide, malonic acid and starch as an indicator; this is the so-called CIMA reaction. When the structure is restricted to a thin layer in a disk reactor, regular striped or hexagonal patterns are formed.

To obtain Turing structures, the species that controls the activation process must diffuse much more slowly than that controlling the inhibitory process. Although all small molecules in aqueous solution have similar diffusion coefficients, the differentials that are needed to generate stationary patterns are obtained in the CIMA reaction by complexation of I_3^- with the large indicator molecules (starch) or with functional groups of the gel matrix — both slow down the activator species⁶.

Analogies

Turing patterns are of interest for several reasons. Turing's original idea was that such patterns might be implicated in morphogenesis. They are also analogous to certain other structures seen in nonlinear physics, such as convective patterns in Rayleigh–Bénard convection, so that the selection of stationary states and the role of defects can be discussed within the same theoretical framework^{7,8}.

Active media support many different kinds of dynamical behaviour, of which Turing patterns are just one manifestation. For instance, all Turing systems can be nudged into oscillatory behaviour. When both the Turing instability and an oscillatory instability occur simultaneously, complex non-stationary structures develop^{9,10}. Turing-type regions and bulk oscillations mix, sometimes creating self-organized structures around localized defects, sometimes purportedly leading to 'chemical turbulence'.

The Texas group³ use a different reaction, involving ferrocyanide, iodate, sulphuric acid and sulphite, in a disk reactor to produce still another kind of non-stationary structure. These structures are generated as follows: a chemical front propagates into a region of different composition, such as a stable state advancing into a less stable one or an excited state into a stable one. With long-range inhibition, of which the Texas system seems to be a striking illustration, the front dynamics change. Fast diffusion makes the inhibitor escape from the front and run ahead of it, hindering its regular propagation until the motion becomes blocked or drastically modified. Then, regions of the system in the same chemical state repel each other strongly over a range comparable to the front width, leading to the appearance of striated structures or blobs, depending on the input concentrations¹¹.

Unlike the Turing instability, where there is long-range correlation between the inhomogeneities, these patterns are well defined locally but disorderly on larger scales. If the proliferation of blobs becomes too great, they can no longer escape repulsive interactions. They must then either become stabilized, so that the fronts no longer propagate, or 'die' by mutual annihilation.

This behaviour was observed previously in numerical models of a reaction-diffusion system¹²; the present work realizes the same phenomena in a real experimental system. At low ferrocyanide concentrations, the Texas group see striated labyrinthine patterns spontaneously generated. As the ferrocyanide concentration is increased, the pattern changes to spiral waves. Finally, they see blobs that sprout off new blobs in a self-replicative process; the blobs die when overcrowding becomes too severe. This is in contrast to the standard development of a Turing spot pattern, which is for spots to emerge in concentric rings — here the growth of a front is limited by inhibitory species, giving rise to new modes of behaviour.

So we now have a bridge between different types of structures: one can readily imagine going from Turing patterns to travelling excitability waves simply by continuously changing a parameter such as temperature or concentration¹³. In fact, some Turing patterns in the CIMA reaction have also been seen to expand by self-replication of blobs rather than in the standard way¹⁰. The new experiments thus form a 'missing link' for a general theory of non-equilibrium patterns, by combining active media with long-range inhibition. As such media are ubiquitous in biology there should be immense interest in further developments in this direction. □

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Rules for Haldane's rule

Jerry Coyne

ONE of the most enduring generalizations in evolutionary biology is Haldane's rule, the principle that if only one sex is sterile or inviable among species hybrids, it is the heterogametic sex. First described in 1922 by the eponymous J. B. S.¹, the rule has received many explanations, but none of them is widely accepted. A paper by H. Allen Orr, however, just published in *Evolution*², proposes a simple explanation that may prove correct.

Haldane's rule is one of the few observations to become an evolutionary 'law', as widely obeyed as the generalization that if only one sex is colourful or ornamented, it is the male. In *Drosophila*, for example, male hybrids are sterile in 112 of 114 hybridizations showing sex-limited sterility, and mammalian hybrids show no exceptions among 25 similar crosses³. Although gender differences in ornamentation are adequately explained by sexual selection, differences in hybrid fertility and viability remain mysterious. It is therefore not surprising that the recent revival of interest in speciation has spawned a fierce competition to explain Haldane's rule.

One clue to a solution is the observation that the rule is also obeyed by birds and lepidopterans^{1,3} (Fig. 1). In these taxa the heterogametic sex is female, and in species crosses it is nearly always the female hybrids which are sterile or inviable. This suggests that, if there is a single cause of Haldane's rule, it has more to do with sex chromosomes than with gender. This notion is buttressed by the many genetic analyses of hybrid sterility and inviability that show a major effect of the sex chromosomes³.

Explanations for Haldane's rule and sex-chromosome effects fall into three categories. The most intuitive is the dominance explanation proposed by H. J. Muller⁴: if some alleles causing sterility or inviability are partially recessive, the heterogametic sex will be more afflicted in F₁ hybrids, for recessive X-linked alleles are expressed more fully in that sex. A second category invokes developmental peculiarities of the X chromosome, including imprinting⁵ or inactivation during spermatogenesis⁶. The third is based on the higher rate of evolution that can occur for some X-linked genes, such as fixation of advantageous recessive alleles⁷ or

meiotic-drive alleles⁸; these genes may subsequently cause problems in heterogametic hybrids.

All of these hypotheses have foundered, however, because they either require unlikely assumptions or fail to explain all of the data³. In consequence, some have proposed that Haldane's rule



FIG. 1. Four of the many species that obey Haldane's rule when crossed to close relatives — *Amadina erythrocephala*, the red-headed finch; *Saturnia pyri*, the great peacock moth; *Cavia fulgida*, the Brazilian cavy; and *Anopheles gambiae*, a mosquito. Two of these species have heterogametic males; two, heterogametic females. (Illustration by Anne P. Crittenden.)

might not have a single explanation and might be only a coincidence produced by different causes in different taxa^{6,9}.

The new theory, which falls into the 'dominance' category, rescues the possibility of a single explanation by adding two simple provisos to Muller's original hypothesis. Orr first points out that Muller's hypothesis not only fails to explain some of the genetic data, but is also theoretically incorrect. Population-genetic calculations show that, although X-linked genes with recessive effects on hybrid sterility or inviability cause more severe problems in heterogametic hybrids, the homogametic sex — by carrying two X chromosomes — suffers from twice as many hybrid incompatibilities involving that chromosome. These two factors exactly balance; thus recessivity itself cannot cause more severe problems in heterogametic hybrids, and fails to explain Haldane's rule.

Muller's explanation can be rescued through Orr's suggestion that hybrids may show a correlation between deleteriousness and dominance, so that alleles causing stronger hybrid sterility or inviability may be more recessive. This correlation would again debilitate heterogametic more than homogametic hybrids. Such a correlation is not a *deus ex machina*, but is exactly what is seen for deleterious alleles

1. Castets, V., Dulos, E., Boissonade, J. & De Kepper, P. *Phys. Rev. Lett.* **64**, 2953–2965 (1990).
2. Ouyang, Q. & Swinney, H. L. *Nature* **352**, 610–612 (1991).
3. Lee, K.-J., McCormick, M. D. & Swinney, H. L. *Nature* **369**, 215–218 (1994).
4. Winfree, A. T. *Scient. Am.* **230**, 82 (1974).
5. Noszticzus, Z., Horsthemke, W., McCormick, W. D., Swinney, H. L. & Tram, W. Y. *Nature* **329**, 619 (1987).
6. Lengyel, I. & Epstein, I. *Proc. natn. Acad. Sci. U.S.A.* **89**, 3977 (1992).
7. Borckmans, P., De Wit, A. & Dewel, G. *Physica A* **188**, 137 (1992).
8. Dufiet, V. & Boissonade, J. *Physica A* **188**, 158–171 (1992).
9. Perraud, J. J. *et al. Phys. Rev. Lett.* **71**, 1272 (1993).
10. De Kepper, P., Perraud, J. J., Rudovics, B. & Dulos, E. *Int. J. Bifurc. Chaos* (in the press).
11. Mikhailov, A. S. *Fundations of Synergetics I* (Springer, Berlin, 1990).
12. Lee, K. J., McCormick, M. D., Ouyang, Q. & Swinney, H. L., *Science* **261**, 192–194 (1993).
13. Pearson, J. E. *Science* **261**, 189–192 (1993).

within species: lethal and sterile mutations are nearly completely recessive in heterozygotes, while less deleterious alleles are more nearly additive¹⁰. (Kacser and Burns¹¹ have explained this well-known observation as the result of loss-of-function mutations affecting enzymes in

report¹³. One would also expect that, because the large 'X-chromosome effect' in crosses is due entirely to recessivity and not to differential rates of evolutionary change, one would see no disproportionate concentration of 'sterility genes' on the X chromosome. There is some evidence

from *Drosophila* that this is true as well (H. Hollocher and C.-I. Wu, personal communication). The proposed recessivity of genes causing postzygotic isolation can be tested by genetically engineering a heterogametic individual that is heterozygous for an X-linked sterility allele and a normal conspecific allele that has been translocated to an autosome. Such a genotype, which could be constructed in *Drosophila*, should recover fertility or viability. Finally, we know almost nothing about the biochemical basis of hybrid sterility or inviability, and Orr's theory must ultimately be tested by working out the genetic and developmental bases of these phenomena.

The most satisfying aspect of the hypothesis is its simplicity: it explains a variety of phenomena with only a few reasonable assumptions, so that one need not invoke different causes of Haldane's rule in

different taxa. Also, by using well-established genetic phenomena within species to explain a pattern discernible only among species, it bridges a gap between micro- and macroevolution. That should appeal to those who believe that speciation can be explained by well-understood principles of evolutionary genetics. □

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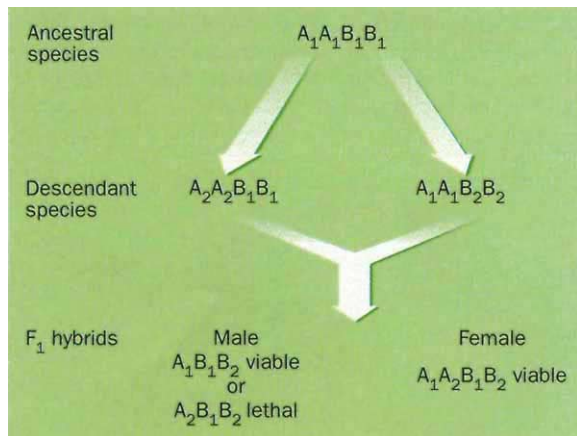


FIG. 2. One possible model for Haldane's rule. In the species involved here, males are heterogametic, having locus A on the X chromosome and locus B on an autosome. An ancestral species fixed for alleles A_1 and B_1 speciates, with one descendant becoming fixed for allele A_2 and the other for allele B_2 . Allele A_2 is inactive on a hybrid (B_1B_2) genetic background. This loss of function at locus A causes hybrid inviability. As shown, male hybrids from one reciprocal cross are lethal because they carry only the inactive A_2 allele. Female hybrids from both crosses have one inactive A_2 allele and one active A_1 allele; according to the model of Kacser and Burns¹¹, these females are viable because the loss-of-function allele A_2 acts as a recessive. Haldane's rule is therefore obeyed in one reciprocal cross. There are many other possible models, which may involve complicated interactions between several hybrid lethal and suppressor genes.

multistep pathways.) Although alleles causing hybrid sterility and inviability must behave normally within species, they could fail to function on a hybrid genetic background. As Dobzhansky and Muller noted half a century ago^{4,12}, these deleterious epistatic interactions involve at least two loci, a 'hybrid lethal' and a 'suppressor'. Figure 2 gives one example of how such interactions might produce Haldane's rule. When combined with the additional proviso (also obeyed within species), that alleles causing sterility usually affect only one sex whereas those causing inviability usually affect both sexes, the new recessivity model explains Haldane's rule in all taxa and the large effect of X chromosomes in heterogametic hybrids.

Orr's theory may, of course, prove incorrect, but it yields several predictions that can be tested. In species obeying Haldane's rule, for example, one would expect on occasion to see reproductive isolation in the homogametic sex in F_2 hybrids, which could then become homozygous for recessive sterility or inviability genes. This phenomenon has been observed, as described in a new

Internal smoke

In much of the Western world, smoking has become a deadly sin. The current social orgy of political correctness and righteous indignation (which Daedalus attributes to widespread sexual and economic frustration) has found smokers to be ideal victims. They cause a highly visible and smellable nuisance, offering splendid scope for moral outrage. Furthermore, they are addicted, and can't just dodge the outrage by giving up. Daedalus now comes to their rescue.

His plan exploits the rechargeable blank drug depot he devised last week. It is injected into the patient, who can then take the matching drug at any time and in any dosage. The depot rapidly absorbs it from his bloodstream, and thereafter leaks it slowly back at the ideal level. It can be recharged by a new dose at any time.

This cunning product was invented for cocaine addicts, to damp their wild highs and stave off their withdrawal lows. It could also deliver long-term drugs such as contraceptives, antihypertensives and antipsychotics. But Daedalus is now targeting it at nicotine, the addictive principle of tobacco. Smoking, he points out, has its own chemical logic. Nicotine by itself is rather unstable: it readily reacts with oxygen. It exists in tobacco as stable salts of citric and malic acids. The heat of smoking breaks these up and releases it as vapour. In the body it soon decomposes, either in the liver or by oxidation. So the smoker has to feed his habit many times a day.

DREADCO's chemists are now devising a fatty depot polymer with acidic groups that react rapidly but reversibly with nicotine. The dissociation constant of this reaction equilibrium will be chosen to leak a low, enduring, stable level of nicotine into the bloodstream: just enough to hold off tobacco hunger.

By itself, the new depot injection will not solve the smoker's problems. It would merely allow him to smoke one enormous cigarette every day, or even every week, and be conspicuously virtuous at all other times. To complete the scheme, Daedalus is devising matching nicotine tablets. Their nicotine is stabilized by acid combination, and when ingested is rapidly transferred to the internal depot by a smooth exchange reaction. By topping up his internal depot with an occasional tablet, the ex-smoker will at last be free of tobacco hunger. He will continue to gain the benefits of smoking, whatever they are, without risk to health or popularity. The tobacco companies will survive as the ultimate source of the nicotine. And the indignation industry will have to look for new victims.

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- Haldane, J. B. S. *J. Genet.* **12**, 101–109 (1922).
- Orr, H. A. *Evolution* **47**, 1606–1611 (1993).
- Coyne, J. A. *Nature* **355**, 511–515 (1992).
- Muller, H. J. *Biol. Symp.* **6**, 71–125 (1942).
- Jablonska, E. & Lamb, M. J. *Proc. R. Soc. B* **243**, 203–208 (1991).
- Wu, C.-I. & Davis, A. *Am. Nat.* **142**, 187–212 (1993).
- Charlesworth, B. *et al.* *Am. Nat.* **130**, 113–146 (1987).
- Frank, S. H. *Evolution* **45**, 262–267 (1991).
- Orr, H. A. *Nature* **361**, 532–533 (1993).
- Simmons, M. J. & Crow, J. F. *A. Rev. Genet.* **11**, 49–78 (1977).
- Kacser, H. & Burns, J. A. *Genetics* **97**, 639–666 (1981).
- Dobzhansky, T. *Genetics and the Origin of Species* (Columbia University Press, New York, 1937).
- Davis, A. W. *et al.* *Genetics* **137**, 191–199 (1994).

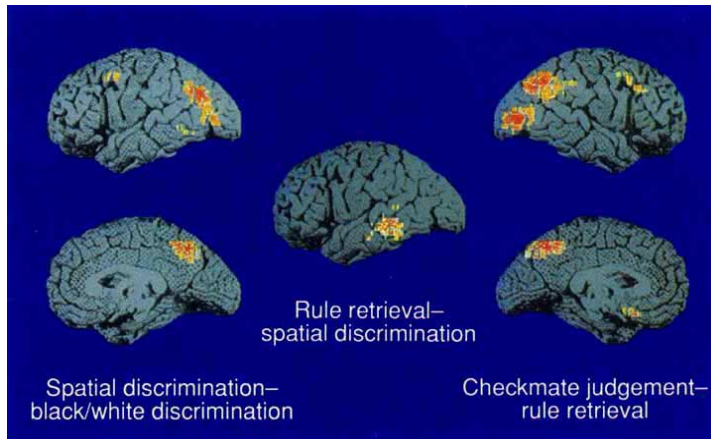
Brain activity in chess playing

SIR — There is widespread interest in identifying the topography of the neural networks subserving problem solving in humans. One potentially fruitful approach is to use functional neuroimaging techniques in conjunction with cognitive tasks¹. Chess is an example of such a task², and we have administered chess problems in conjunction with [¹⁵O]water-positron emission tomography (PET) to identify the neural network(s) activated in this process³.

The stimuli for all the task conditions were black-and-white chess diagrams presented on a computer screen. To isolate brain activation changes related to the use of specific cognitive processes, we devised four conditions arranged in a subtractive hierarchy: (1) black/white discrimination, in which subjects had to indicate whether or not there were chessmen of a given colour on the board; (2) spatial discrimination, in which an *X* was displayed in one of the board's squares and subjects had to identify the colour of the chessman closest to the *X*; (3) rule retrieval, in which subjects analysed a simple, single move, in responding to questions like "can the white knight capture a black rook?"; and (4) checkmate judgement, in which the subjects had to determine whether the player with a given colour could checkmate in one move. All answers were indicated by pressing response keys labelled yes or no. Ten right-handed males who had played chess for more than 4 years and were regularly participating in chess tournaments volunteered for this study.

When the results of the black/white discrimination condition were subtracted from the spatial discrimination condition, brain activation was observed bilaterally (see figure for a visual rendering of all the analyses) at the parieto-occipital lobe junction (areas 7 and 19), at the left middle temporal gyrus, and at the left superior premotor cortex. Extrastriate visual cortical areas are mainly organized around two anatomically separate and functionally specialized processing streams: a ventral occipito-temporal pathway for identifying objects; and a dorsal occipito-parietal pathway for perceiving the spatial relations between objects^{4,5}. The black/white discrimination task only

required the subject to process figure-ground separation and discriminate between black and white chess pieces. The extra visual analysis required by the spatial discrimination condition explains the dorsal pathway activity. Activity in area 7 (both in the superior parietal lobule and in the medial superior parietal cortex) has



Cerebral regions associated with cognitive components resulting from subtraction between the four tasks. PET images were reconstructed by bilinear interpolation in the axial (z) dimension to produce transaxial planes. Data were displayed in a 128×128×43 voxel matrix, each voxel having similar dimensions in each of the three axes. Any roll and/or yaw misalignment between scans was corrected based on visual inspection independently by P.N. and P.P. For each comparison an image of the pixel t-values was created, constituting the statistical parametric map (SPM{t}). The omnibus significance of these t-statistical parametric maps was assessed by comparing the expected and observed number of pixels above a significance threshold of $P=0.001$ (ref. 9). Only SPMs that were significant in the omnibus sense (at $P<0.001$) are reported. To illustrate the distribution of the main sites of activation, significant areas of activation for each subtraction condition are rendered on the lateral and (when appropriate) the medial surface of the brain used in ref. 10.

been repeatedly associated with spatial vision and with shifting spatial attention. We also found activation in a region of the left superior premotor cortex (superior area 6) which previous studies suggested was related to preparing a response to a selected peripheral location.

Subtraction of the spatial discrimination condition from the rule retrieval condition yielded several foci located

along the lateral, the inferior, and the medial aspects of the left temporal lobe, the latter being mainly due to activation of the left hippocampus. We suggest that the retrieval of move sequences paired with a particular chess piece was responsible for the hippocampus and temporal lobe activation⁶. A small peak of activation was also found in the inferior part of the post-central gyrus as well as in the cerebellum, which has recently been implicated in cognitive processing⁷.

In the subtraction of rule retrieval from checkmate the differential activity was confined to two large areas, the junction between the occipital and the parietal lobe, and the frontal lobe. We attribute the bilateral focus of activation we recorded at the occipito-parietal junction (areas 7, 18 and 19) to the act of generating sequential board images and to attentional shifts across the images. Activation recorded in the frontal eye fields (area 8) might be associated with an increase in extraocular movements during the instantiation of various endgame strategies. We also found increased activity in two prefrontal regions, one located in the left orbitofrontal cortex, and the second in the right prefrontal cortex. We believe that these two regions of activation subserve managerial knowledge required for the planning and sequential execution of endgame strategies⁸.

We have demonstrated that solving a complex problem calls for the activity of a network of several interrelated, but functionally distinct, cerebral areas. The use of neuroimaging techniques such as PET, carefully coupled with distinctive information-processing demands, can help disentangle the role of each of these areas in human problem-solving activity.

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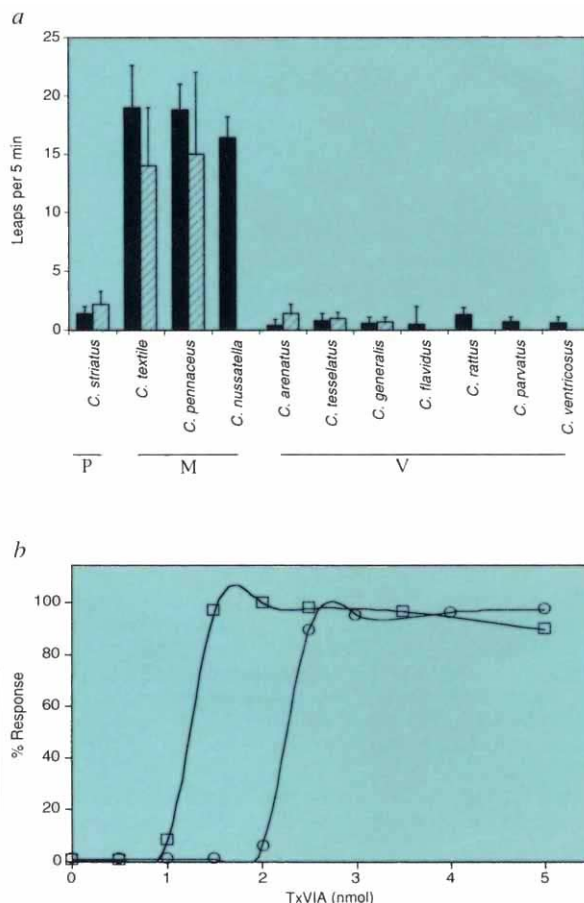
1. Roland, P. E. & Friberg, L. J. *Neurophysiol.* **53**, 1219–1243 (1985).
2. Charness, N. in *Towards a General Theory of Expertise: Prospects and Limits* (eds Ericsson, K. A. & Smith, J.) 39–63 (Cambridge University Press, 1991).
3. Raichle, M. E. in *Handbook of Physiology, Section 1: The Nervous System* **5**, 643–674 (1987).
4. Desimone, R. & Ungerleider, L. G. in *Handbook of Neuropsychology* (eds Boller, F. & Grafman, J.) 267–299 (Elsevier, Amsterdam, 1990).
5. Haxby, J. V. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 1621–1625 (1991).
6. Milner, B. *Br. med. Bull.* **27**, 272–277 (1971).
7. Fiez, J. A., Petersen, S. E., Cheney, M. K. & Raichle, M. E. *Brain* **115**, 155–178 (1992).
8. Grafman, J. in *Integrating Theory and Practice in Clinical Neuropsychology* (ed. Perecman, E.) 93–138 (Erlbaum, Hillsdale, New Jersey, 1989).
9. Friston, K. J., Frith, C. D., Liddle, P. F. & Frackowiak, R. S. J. *Cereb. Blood Flow Metab.* **11**, 690–699 (1991).
10. Talairach, J. & Tournoux, P. *Co-planar Stereotaxic Atlas of the Human Brain* (Thieme, Stuttgart, 1988).

Marine warning via peptide toxin

SIR—Chemosensory signals are crucial in the mediation of animal behaviour in marine ecosystems^{1,2}. The vast dilutions and the diversity of background chemical noise produce stringent requirements from a selective chemical signal in aquatic environments². Peptides have been suggested as ideal candidates for such specific

ated by distance chemoreception and can be induced by conditioned water from venomous *Conus* snails^{5,6}. Because *Conus* snails are highly specialized in their feeding ecology⁷, we surmised that an ability to differentiate chemically between molluscivorous and non-molluscivorous Conidae should be selectively advantageous for *Strombus*. A scrutiny of conditioned water from 11 species of *Conus* revealed that only that of three molluscivorous species elicited an escape response in *S. gibberulus* (*a* in the figure), thus confirm-

a, Reaction of adult *S. gibberulus* to application of conditioned water from 11 species of Conidae (black bars), or to venom peptide fractions from six species of Conidae (hatched bars). Conditioned water was tested by placement of five strombs in the centre of the container used for the overnight conditioning by the cones. Venom peptides (10 mg fraction protein in 100 μ l ASW) were tested by application adjacent to a stromb in a container of 1 litre clean ASW. Stromb reactions were quantified by counting the total number of leaps performed by each individual animal within five minutes of application of the test substance. Mean $\pm$ s.d. for 3 replicates of each experiment is shown. The Conidae tested include one piscivorous species (P), three molluscivorous species (M) and seven vermivorous species (V). *b*, Synergism between conotoxin-TxVIA and conditioned water in eliciting *Strombus* escape response. Increasing doses of TxVIA were applied together with a subthreshold level of conditioned water (squares), or 5 min after exposure (circles). Each data point is the average of two replicates.



cues, owing to the high information content inherent in their structures, and their solubility in water³. Here we show that conotoxin-TxVIA, a phylogenetically selective peptide neurotoxin⁴, is exploited as a specific warning cue by a prey snail of the genus *Strombus*, thus demonstrating that a peptide of known structure acts as an interspecies chemosensory cue in the marine environment. This finding represents an intriguing situation whereby the selectivity of a peptide allomone is used by the target prey as a specific kairomone, and suggests parallels in chemical communication between neurons and in marine ecosystems.

Certain marine snails react to danger by stereotyped escape behaviour: *Strombus* snails perform spectacular, 'kangaroo-like' leaping movements when confronted with predators. This behaviour is medi-

ated by distance chemoreception and can be induced by conditioned water from venomous *Conus* snails^{5,6}. Because *Conus* snails are highly specialized in their feeding ecology⁷, we surmised that an ability to differentiate chemically between molluscivorous and non-molluscivorous Conidae should be selectively advantageous for *Strombus*. A scrutiny of conditioned water from 11 species of *Conus* revealed that only that of three molluscivorous species elicited an escape response in *S. gibberulus* (*a* in the figure). A comparison between *C. textile* conditioned water and its venom revealed very different compositions, although venom components are also released to the surroundings by Conidae, as droplets of venom released while searching for or

ing that this species can discriminate solely on the basis of chemical stimuli between potentially dangerous and non-dangerous species from the same predatory genus. This result suggested the existence of phylogenetically specific alarm cues in Conidae. As certain molluscivorous *Conus* venom peptides are specifically toxic to molluscs⁴, we tested six *Conus* venoms to see if they could fulfil the role of specific alarm cues recognized by the *Strombus*. Only the molluscivorous species venoms were active, although less consistently than their conditioned water (*a* in the figure). A comparison between *C. textile* conditioned water and its venom revealed very different compositions, although venom components are also released to the surroundings by Conidae, as droplets of venom released while searching for or

We therefore examined the effects of coapplication of conotoxin-TxVIA from *C. textile* with its conditioned water. TxVIA is specifically paralytic to molluscs⁴ and slows inactivation of voltage-dependent sodium currents^{8,9}. The toxin alone at doses of up to 20 nmol did not elicit any response from adult *Strombus* (our unpublished data), but, when applied in the presence of a subthreshold level of *C. textile* conditioned water, the combination caused a spectacular reaction in *Strombus* (*b* in the figure). There was no reaction of the test *Strombus* to combinations of *C. textile* conditioned water with peptide fractions from three other non-molluscivorous *Conus* venoms, or to application of 10 nmol of heat-inactivated TxVIA together with conditioned water (our unpublished data). There is a clear dose-dependence in the triggering of the reaction, with the threshold effective dose between 1.5 and 2.5 nmol (*b* in the figure). Taking into account the dilution volume, the effective concentration of TxVIA under these conditions is in the same range as that required for its pharmacological effects^{8,9}. (Note that these data do not enable a distinction between TxVIA exerting its effects on the *Strombus* via sodium channels, or via unrelated chemosensory receptors.)

We also performed field experiments to simulate a situation whereby a droplet of *C. textile* toxin is released by a hunting cone in the vicinity of strombs in their natural environment. *S. gibberulus* were placed 7 m downstream of large *C. textile* and observed for 15 min, during which time no unusual movements were observed. After 15 min the *Strombus* were exposed to 7 nmol TxVIA applied nearby. Two-thirds of the experimental animals immediately began violent bursts of jumping.

The data presented in this report suggest that a venom-derived peptide is a specific alarm cue for prey snails. *Strombus* can distinguish between molluscivorous and non-molluscivorous *Conus* snails, their escape reaction is enhanced by a synergic mix of venom-derived peptides and other metabolites, is dose-dependent in a threshold 'all-or-none' manner and is apparently specific for bioactive conformations of the venom peptide. Specificity of the *Strombus* escape response

1. Carr, W. E. S. in *Sensory Biology of Aquatic Animals* (eds Atema, J. et al.) 3–27 (Springer, New York, 1988).
2. Morse, D. E. *Bull. mar. Sci.* **46**, 465–483 (1990).
3. Rittschof, D. & Bonaventura, J. *J. chem. Ecol.* **12**, 1013–1023 (1986).
4. Fainzilber, M. et al. *Eur. J. Biochem.* **202**, 589–595 (1991).
5. Gonor, J. J. *Veliger* **8**, 226–230 (1966).
6. Kohn, A. J. & Waters, V. *Anim. Behav.* **14**, 340–345 (1966).
7. Kohn, A. J. & Nybakken, J. W. *Mar. Biol.* **29**, 211–234 (1975).
8. Hasson, A. et al. *Eur. J. Neurosci.* **5**, 56–64 (1993).
9. Fainzilber, M. et al. *J. Biol. Chem.* **269**, 2574–2580 (1994).

might be ensured by the requirement for a high concentration of a cocktail of specific toxins or a synergic combination of a toxin together with other *Conus* metabolites. We have demonstrated the ecological relevance of this phenomenon in field experiments. This is, to the best of our knowledge, the first documented case of a defined peptide acting as an interspecific alarm cue in marine ecosystems. In the ecological interaction described herein, a prey animal is able to turn the tables on its selective predator by taking advantage of its attack allomones as specific warning signals.

Another intriguing aspect is the implication of structural and functional similarities between chemosensory receptors and nervous system receptors. Such similarities might have provided *Strombus* with a preadaptation, whereby any selective chemical targeted against a receptor in its nervous system would be specifically recognized by the related chemosensory receptors.

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Next step for weekday warming

SIR — The assertion¹ that the lower troposphere (1,000–400 hPa, or about 0–7,000 m) shows a weekly periodicity in temperature is surprising in view of available estimates of human energy consumption and its direct impact on the temperature of the lower troposphere. The world energy consumption per unit area for 1992 has been estimated at $7.2 \times 10^5 \text{ J m}^{-2} \text{ yr}^{-1}$ (ref. 2).

On the conservative assumption that this consumption occurs solely in the Northern Hemisphere, this amounts to about 0.045 W m^{-2} . If all this energy is transformed into sensible heating of the lower troposphere, this leads to a heating rate of $6.4 \times 10^{-4} \text{ K per day}$, or only about 9% of the observed maximum rate of increase seen in the weekly temperature cycle¹, which shows a heating rate of about 0.02 K in 3.5 days, or 0.006 K per day for the Northern Hemisphere.

The energy rate required to produce this observed change in temperature is 0.40 W m^{-2} . Therefore, even if human energy consumption were turned off long enough for the atmosphere to come to equilibrium, then suddenly turned on, the

rate of temperature increase would be about an order of magnitude smaller than that observed.

For comparison, it is interesting to estimate how much the Earth can heat up owing to human energy consumption, assuming the Earth would reach a new radiative equilibrium and that all the energy consumption is transformed into sensible heating of the atmosphere (neglecting change in the Earth's surface temperature). The amount of infrared radiation emitted by the atmosphere to space is around 155 W m^{-2} (ref. 3). Assuming the total human energy consumption is used to increase the radiative temperature of the Northern Hemisphere atmosphere leads to a steady-state temperature increase of about 0.02 K. (The same assumptions applied only to US energy consumption lead to a temperature increase over the conterminous United States of 0.15 K.) Thus, the problem is not that the magnitude of the observed temperature change is obviously unreasonable, but that the rate of change is too large to be accounted for directly by human energy consumption.

Before dismissing this suggestion, it may be worth investigating other physical processes that might be triggered by weekly variations in human activities. Certainly variations do occur in, for example, vehicle use, power-plant production, and agricultural and recreational activities during the week. Therefore, it is likely that human activities do impose a weekly cycle on aerosol production and distribution, with higher production during the week. This could alter the solar heating or infrared cooling rate of the lower troposphere, or the precipitation rate, which in turn could modify the temperature of the lower troposphere.

Some of the terms in the Earth's radiation budget that might be affected are^{3,4}: backscattered solar radiation, 21 W m^{-2} (2); solar radiation absorbed in the clear atmosphere, 56 W m^{-2} (0.7); solar radiation reflected by clouds, 70 W m^{-2} (0.6); solar radiation absorbed by clouds, 14 W m^{-2} (2.9); and infrared radiation emitted by clouds, 91 W m^{-2} (0.4). (Numbers in parentheses are the percentage magnitudes of the weekly oscillation in these processes averaged over the entire Northern Hemisphere which could lead to the observed temperature change.) Similarly, the latent energy rate released by precipitation averaged over the Northern Hemisphere (estimated⁵ to be 1 m yr^{-1}) is 79 W m^{-2} (0.5).

Although the per cent changes required in these processes are small, it is not obvious that they could be modulated on timescales of a week even if human activities are modulated on a weekly basis. Furthermore, the maximum of the microwave radiometer weighting function is in the range of 500–700 hPa (ref. 5), above

the usual height of the atmospheric boundary layer, and contributions from heights greater than 400 hPa are not insignificant. Because coupling between the boundary layer, where anthropogenic aerosols are predominately produced, and the air above is episodic and of the order of several days, it is likely that only boundary-layer aerosols can be modulated significantly on a weekly cycle. As pointed out above, their direct impact on the global radiation budget is less than for clouds. Therefore, it is not likely that the observed temperature changes are directly a result of aerosol impact on radiation.

At first glance, clouds seem to be a more likely candidate. They commonly incorporate boundary-layer air, and changes in aerosols can have significant impact on the microphysical structure of clouds, which can affect both solar and infrared radiation and rainfall rates^{6,7}. But the impact of increased aerosols is likely to be increased albedo and decreased rainfall, which would result in cooling during the week and warming at weekends. Therefore, there seems to be no plausible physical mechanism that could account for the observed midweek temperature maximum.

But it is more likely that the observations are not statistically significant. Because the results are not significant at the 5% level, this would seem to warrant further testing. For example, is there any significance if the time series are split into two samples? What is the statistical distribution of the means? Examples of the microwave temperature data show monthly and seasonal variations⁵. How does this affect the significance of the weekly temperature variation estimates?

To investigate further the possibility of a weekly periodicity, is it possible to look for weekly variations in hemispheric averages of other variables such as the solar reflectivity (albedo), infrared radiation, or rainfall rate of the Earth? Admittedly, the magnitudes of the changes would be small, but if they can be found, they would lend credence to the observation of a weekly temperature cycle in the Northern Hemisphere, and would suggest the possibility of deducing anthropogenic effects before climate change had occurred.

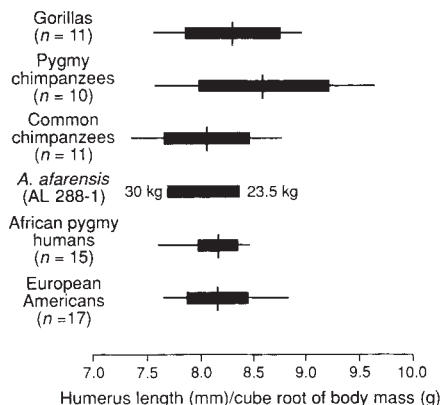
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- Gordon, A. H. *Nature* **367**, 325 (1994).
- World Almanac and Book of Facts* (Funk & Wagnalls, 1994).
- Sellers, W. D. *Physical Climatology* (University of Chicago Press, 1965).
- Peixoto, G. P. & Oort, A. H. *Physics of Climate* (American Institute of Physics, 1992).
- Spencer, R. W., Christy, J. R. & Grody, N. C. *J. Clim.* **3**, 1111–1128 (1990).
- Liou, K. N. *Radiation and Cloud Processes in the Atmosphere* (Oxford University Press, 1992).
- Albrecht, B. A. *Science* **245**, 1227–1230 (1989).

Ape and hominid limb length

SIR — The new discoveries of *Australopithecus* at Maka in Ethiopia¹ are important additions to the fossil record of early hominid evolution. Among these finds, an incomplete, “very robust” humerus “from a large, presumably adult male” (MAK-VP-1/3) figures prominently in inferences about locomotor capabilities of *A. afarensis*^{1,2}. White *et al.* conclude that this



Relative humerus length in African apes, modern humans and *A. afarensis*. The mean is represented by the vertical line; ± 1 s.d. is indicated (solid bars) along with minimum and maximum values.

basal hominid “retained a powerful upper limb, but an upper limb that lacked the key arboreal adaptation of great length”¹. Gee in News and Views² suggests this means that the humerus of *A. afarensis* is “relatively short” and “not what one would expect of a tree-living creature”.

The functional significance of relative limb lengths in *A. afarensis* has been debated for over a decade^{3–6}. The key word here is *relative*; absolute lengths are unrelated to locomotor performance (for example, the highly suspensory gibbon has absolutely shorter humeri than do humans or gorillas). I pointed out^{3,5} that the relative humerus length of *A. afarensis* is similar to modern humans, and that “Lucy” (AL 288-1) cannot be distinguished from pygmy chimpanzees or pygmy humans in this regard. I have combined these data with new observations (see figure) to reassess the relationship between relative humerus length and locomotor repertoires in *A. afarensis*, modern humans and African apes. All species overlap extensively in relative humerus length, with pygmy chimpanzees possessing the longest, on average, and common chimpanzees the shortest. A Kruskal–Wallis test of the five extant samples indicates that there are no significant differences in this index ($P > 0.12$). Model II log–log regression of humerus length on mass confirms this result; the

scaling relationship is best described as isometric (for the sample as a whole and for African apes alone). Put simply, arm length relative to body size cannot discriminate by itself among species that are adept arborealists (pygmy and common chimpanzees), primarily terrestrial but capable climbers (gorillas) and habitually terrestrial bipeds (humans).

In my view, the conclusions reached by White *et al.*¹ and Gee² are not supported. The relative length of the humerus in *A. afarensis* might be precisely the length one would expect of a species at home both on the ground and in the trees. Other aspects of the postcranial skeleton that reflect or facilitate arboreal behaviours are consistent with this interpretation^{7,8}. It is also indisputable that *A. afarensis* exhibits skeletal traits linked to terrestrial bipedalism, but terrestriality does not necessarily preclude arboreality.

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WHITE REPLIES — Jungers speculates that *A. afarensis* was “at home both on the ground and in the trees” because a ratio of humeral length to body mass does not discriminate among African apes, humans and *A. afarensis*. Such a test might be appropriate if these taxa did not differ in other crucially important locomotor characters. However, they do differ dramatically.

First, Jungers has elsewhere argued that humeral length is negatively allometric with increasing body mass^{3,9}. He now concludes that it is isometric. Which is correct?

Second, why has Jungers excluded hylobatids and orangutans from his sample? Elsewhere he has described these *arboreally adapted* hominoids as having “pronounced *relative* forelimb elongation”⁹ (emphasis added).

Third, the African apes are knuckle-walking terrestrial quadrupeds and some-

times arboreal feeders (*Pan troglodytes* averages 84 per cent of total locomotor activities on the ground¹⁰). Chimpanzees are agile arborealists largely because they rely on flexible, grasping hindlimbs with opposable great toes. The hindlimb of *A. afarensis* was extensively specialized for bipedality, with loss of virtually all grasping ability. Would it not exhibit morphology consistent with other hominoids whose forelimbs are also uncoupled from knuckle-walking if it were “at home in the trees?” MAK-VP-1/3 is an ideal test of this locomotor hypothesis. As noted by Gee, it lacks any such morphology. It is both retroflexed (like only humans among hominoids) and short by any arboreal (not knuckle-walking) standard.

Since leopards, lizards, snakes, baboons and modern humans can all climb trees, we agree with Jungers that “terrestriality does not necessarily preclude arboreality”. At issue, however, is how to warrant our inferences about Pliocene hominid activities. The total morphological pattern of the *A. afarensis* locomotor skeleton includes the *relatively* shortest manual (and pedal) phalanges of any primate except *Homo sapiens*; a completely transformed hip abductor apparatus; full lumbosacral lordosis; habitual full extension of the knee; a reorganized ankle joint with horizontal plafond; and a foot with adducted hallux and longitudinal and transverse arches^{6,11–15}. As Gee points out, it seems inappropriate to suggest that a short, retroflexed, human-like humerus represents evidence for ape-like arboreality in such a species.

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21st century read

SIR — For Guy Hewlett’s comment (*Nature* 368, 697; 1994), that there is a 95% probability that we will be reading correspondence relating to Gott’s over the next 30.6 years, to be credible in Gott’s terms, one has to accept Gott’s assumptions (the Copernican view). Those reading this Scientific Correspondence, as well as the original Hypothesis, now have a different Gott probability time period to contend with to that suggested by Hewlett.

Criticism so far of Gott’s Hypothesis largely springs from the interpretation and recognition (or lack of) of his assumptions — a largely fruitless game we can all play. And so, in Machiavellian gratification of my human desire for notoriety, I have placed my travelling atomic alarm clock in a box marked ‘Gott’, set for AD 2024, with instructions to write to *Nature*.

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- White, T. D. *et al.* *Nature* **366**, 261–265 (1993).
- Gee, H. *Nature* **366**, 207 (1993).
- Jungers, W. L. *Nature* **297**, 676–678 (1982).
- Wolpoff, M. H. *Nature* **304**, 59–61 (1983).
- Jungers, W. L. in *Origine(s) de la Bipedie chez les Hominides* (eds Coppens, Y. & Senut, B.) 215–224 (Editions du CNRS, Paris, 1991).
- Latimer, B. in *Origine(s) de la Bipedie chez les Hominides* (eds Coppens, Y. & Senut, B.) 169–176 (Editions du CNRS, Paris, 1991).
- Susman, R. L., Stern, J. T. Jr & Jungers, W. L. *Folia Primatol.* **43**, 113–156 (1984).
- Hunt, K. J. *J. hum. Evol.* **26**, 183–202 (1994).
- Jungers, W. L. in *Size and Scaling in Primate Biology* (ed. Jungers, W.) 345–381 (Plenum, New York, 1985).
- Doran, D. M. *Am. J. phys. Anthropol.* **91**, 99–115 (1993).
- Latimer, B. & Lovejoy, C. O. *Am. J. phys. Anthropol.* **78**, 369–386; **82**, 125–134; **83**, 13–23 (1989–90).
- Lovejoy, C. O. *Sci. Am.* **256**, 11:118–125 (1988).
- Latimer, B., Ohman, J. C. & Lovejoy, C. O. *Am. J. phys. Anthropol.* **74**, 155–175 (1987).
- Johanson, D. C. *et al.* *Am. J. phys. Anthropol.* **57**, 373–724 (1982).
- White, T. D. & Suwa, G. *Am. J. phys. Anthropol.* **73**, 485–515 (1987).

A mind in motion

Owen Gingerich

Galileo: Decisive Innovator. By Michael Sharratt. Blackwell: 1994. Pp. 297. £19.99, \$29.95.

OF all the scientists delineated by biographers, Galileo ranks as a perennial favourite along with Darwin and Einstein. There are more twentieth-century biographies in English of Galileo than of Copernicus, Kepler, Hubble, Shapley and Eddington combined. In recent years, Stillman Drake, Pietro Redondi and Mario Biagioli have produced thematic biographies, while Richard Westfall, Richard Blackwell and Michael Segre have produced important ancillary studies; at least two more biographies are in the pipeline. Do we need yet another?

In approaching Galileo afresh, Michael Sharratt demonstrates a thorough familiarity both with the vast Galilean corpus preserved in the 20-volume so-called "National Edition" produced at the end of the last century and with much of the contemporary literature; and, uniquely, he provides a critical analysis of the current Vatican attempts to rehabilitate the seventeenth-century astronomer who, in Cardinal Poupard's words "had much to suffer". In a sense, Sharratt's is almost a metabiography, for he discusses not only Galileo, but what other recent biographers have been saying about him.

Galileo: Decisive Innovator begins at the provocative fulcrum of Galileo's career, with the application of the as yet unnamed spyglass to the heavens, with the publication of the *Sidereus nuncius* (1610) containing its startling views of the Moon and of Jupiter's satellites, and with Galileo's now-firm commitment to the Copernican heliocentric cosmology. Copernicanism becomes the central theme, as Sharratt shows how Galileo's work in both astronomy and physics was devoted to transforming "the obviously ridiculous into the ridiculously obvious". Although Galileo's contributions to mechanics are prominently displayed (including the work before 1610), they are always subordinated to the central intellectual focus of Galileo's cosmology. In other words, this is a well-framed account of how Galileo got in trouble with the Inquisition, but instead of depicting only the clash of personalities or the drama that led to Galileo's trial, Sharratt paints from a far wider palette, giving the scientific ideas their full due and describing each of Galileo's works in comfortable detail.

Sharratt is at his best when he discusses Galileo's attitude towards the interpretation of scripture. In a nuanced account,

he shows that Galileo was primarily concerned to prevent an ill-judged condemnation of Copernican cosmology, but he undermined his own position by saying that if a proposition is not proved conclusively and contains something contrary to scripture, then it is to be reckoned undoubtedly false. Because Galileo had not, in fact, been able to provide a cogent proof of heliocentrism, and because many churchmen could cite "proof texts" for an immovable Earth, Galileo had "sawn off the branch he was sitting on". Obviously Galileo did not see it that way, and dismissed the scriptural citations as mere colloquial language. As Pope John Paul II has said, "Galileo showed himself to be more perceptive in this regard than the theologians who opposed him".

The fact that Sharratt is a Roman Catholic priest as well as a lecturer in philosophy at the University of Durham

may account for his selection of material, but a reader could hardly discern this fact from a close examination of the book. He is critical of Rome's handling of the matter in the seventeenth century as well as in the twentieth. In a few minor details of historical interpretation I would personally have sketched the scene differently. But altogether this is an unusually well-balanced account.

The book is illustrated throughout with historical black-and-white graphics carefully chosen to reproduce well. I was astonished, however, to note that none of Galileo's first editions is included, and there is only one mediocre late picture of Galileo himself. It is as if the author relied on a provincial library that could offer some rarely seen curiosities without engaging visually with the central works that are so carefully analysed in the text itself. Despite this shortcoming, the book is impressive, and it goes to the top of my list for anyone wanting a comprehensive introduction to Galileo, both the man and his innovations. □

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YOUNG marabou storks (*Leptoptilos crumeniferus*), from *Okavango: Africa's Last Eden* by Frans Lanting. The book's photographs, which won the author the title of BBC Wildlife Photographer of the Year in 1991, provide a rich and original portrait of the flora, fauna and landscape of this 8,500-square-mile wetland. A spectacular browse. Robert Hale, £27.99.

Silent upon a peak in Darien

Stuart Ross Taylor

Exploring Planetary Worlds. By David Morrison. W. H. Freeman: 1994. Pp. 240. £19.95, \$32.95.

Planetary Landscapes. 2nd edn. By Ronald Greeley. Chapman & Hall: 1994. Pp. 286. £79.50, \$99.95 (hbk); £27.95, \$49.95 (pbk).

Venus: The Geological Story. By Peter Cattermole. UCL Press/Johns Hopkins University Press: 1994. Pp. 250. £25, \$39.95.

THE study of planetary landscapes, the topic of these three books, opens new vistas to previously Earth-bound students of geomorphology. The study of extra-

Apart from the telescopic views of the Moon, and the enigmatic fuzzy images of Mars available to previous generations, we are the first generation to view these worlds. Like "stout Cortez . . . and all his men . . . silent, upon a peak in Darien" as they stared at the vast Pacific, one views these freshly revealed and incredible landscapes with awe; mountains that dwarf Everest, canyons into which the Grand Canyon of Colorado could vanish, "deserts of vast eternity", icy landscapes simulating those due to terrestrial lavas, and the ubiquitous craters, like some gigantic cosmic version of a battlefield.

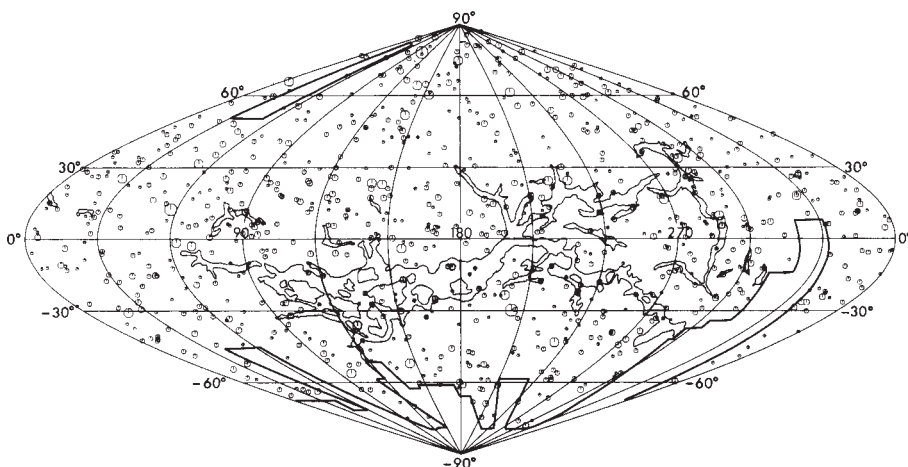
All are so different from our familiar landscapes that they could belong to planets orbiting around another star without exciting comment. These observations raise fundamental philosophical questions, not addressed by the authors, about the uniqueness of the Earth. No two planets or satellites are alike. Even the

planetology is that terrestrial analogues are a trap. So when Cattermole notes that "some of the Venera data point to considerable differentiation of the Venusian crust to form *continental* material akin to that so typical of the Earth", readers should note that a few scattered 'pancakes' of viscous lava, about 25 km in diameter, represent the only material apparently more siliceous than basalt on the Venusian surface. The Australia-sized Ishtar Terra and other highlands are probably crumpled-up basalt, not granite, which may be a unique product of our own wet tectonic system.

In this context, there is little in these books for geochemists interested in planetary compositions or geophysicists wondering about planetary interiors; these are descriptive texts about the surfaces of the solid planets and satellites, a necessary first step to any deeper understanding. Morrison gives a very readable semipopular overview suitable for the educated lay public, Greeley provides a text for senior undergraduates and graduate students interested in geomorphology, while Cattermole deals exclusively with Venus, providing the first geology text for that planet. The technical texts of Cattermole and Greeley should be in all Earth and planetary science libraries; both are suitable for course work, with Greeley's text being at a more advanced level. Cattermole's book makes an interesting contrast to Henry Cooper's *The Evening Star: Venus Observed* (Farrar, Straus and Giroux, 1993; for a review see *Nature* 364, 683; 1993), which describes the activities of the Magellan science team, struggling to make sense of the data as they arrived in staggering quantity; an interesting account of science being done in "real time".

The reproduction of the images in all three books is among the best I have seen, but scales, essential information when looking at planetary images, are missing on nearly half the Magellan images in Cattermole's book, and on many of the images in the first half of Greeley's; the situation improves in the second half of the latter text.

A few errors, seemingly inevitable on such a broad canvas, have crept in. Greeley gives a rotation rate for Venus of 116 days, rather than 243 days, so that the planet in fact takes longer than an orbital period (225 days) to rotate. He also states that Uranus and Neptune are, like Jupiter and Saturn, "composed of hydrogen and helium", whereas they are mostly ices (of water, ammonia and methane) with only about 10 per cent gas. Morrison repeats the familiar and deep-rooted error that the Moon is derived from the mantle of the Earth, although all the computer simulations of that dramatic event derive our satellite from the rocky mantle of the impactor, thus accounting for the signifi-



Magellan map of 842 impact structures on the surface of Venus. From *Venus*.

terrestrial landscapes started in 1609 with Galileo's observation that "the Moon certainly does not possess a smooth and polished surface", so opening the possibility that other worlds might look "just like the face of the Earth itself". For the next three centuries, debate continued over the nature of the lunar surface, and was heavily biased towards familiar terrestrial examples. It was only in the 1960s that the correct interpretation of the lunar craters as due to meteorite impact rather than volcanism was generally accepted, vindicating the perceptions of G. K. Gilbert in 1893 and Ralph Baldwin in 1949. Until the advent of space exploration, however, such questions were peripheral to geomorphologists, who had a plethora of terrestrial landforms to account for and who were preoccupied with peneplains, erosion rates and landscape cycles. Now, the new vistas from the past three decades of exploration of the Solar System have provided a flood of fresh geographic information, unrivalled since Elizabethan times.

What a feast of scenes is revealed.

surface of Venus, close in size, mass, density and probably bulk composition to the Earth, is recognizable as related only in a Jekyll-Hyde sense. If it is so difficult to produce clones within this planetary system then the chances of producing them in another system seem as remote as the chance of repeating the evolutionary sequence that led to *Homo sapiens*.

Venus is now our best explored planet (the topographic information on the Venusian surface outranks that on our own ocean floors) owing to the remarkable quality of the radar images from Magellan, a mission that has returned more data than all previous space missions combined. The final story on Venus is not yet in: the vexing question whether volcanism and hence planetary resurfacing shut down a few hundred million years ago remains a hotly debated question; possibly Venus has formed an impervious hide, like an armadillo, and, in the absence of plate tectonics, recycling and subduction, the planet has choked itself on basalt.

One of the lessons of comparative

cant differences in composition between the terrestrial mantle and the Moon.

Cattermole and Greeley both provide comprehensive lists of references; surprising omissions in Greeley's bibliography are Jay Melosh's standard text *Impact Cratering: A Geologic Process* (Oxford University Press, 1989) and Don Wilhelm's marvellous account of lunar stratigraphy (US Geological Survey Professional Paper 1348, 1986).

The overall effect is stunning, however. What student of W. M. Davis, Charles Cotton or Bill Thornbury would have imagined having the landscapes of a multi-

tude of new worlds to investigate? The differences among the planets and satellites bring another sobering message: if we make this planet uninhabitable, or if doom threatens from an asteroid or cometary impact, only Mars might serve as a refuge. It would be slightly more comfortable, assuming an oxygen supply, than Antarctica, but with the blizzards replaced by global dust storms. □

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Knowing the technological future

David Edgerton

Exploring the Black Box: Technology, Economics and History. By Nathan Rosenberg. Cambridge University Press: 1994. Pp. 274. £40, \$54.95.

THE generation of new scientific and technological ideas and artefacts is, in the late twentieth century, a huge industry in its own right. It absorbs two per cent and more of the gross domestic product of the richest industrial countries. This industry, like so many others, has attracted the attention of economists and historians and, because so much of it is publicly financed, students of government too. Indeed, the innovation industry has been regarded as more important, and more characteristic of the modern age, than any other. After all, for Alfred Whitehead, the greatest invention of the nineteenth century had been invention itself. For Joseph Schumpeter, what he saw (in the interwar years) as the 'routinization' of innovation made obsolete the key historical figure of the entrepreneur and paved the way for socialism (of a sort). For others, such as J. D. Bernal, planned invention and development required socialism, and promised abundance and social harmony.

If sulphuric acid production was once a proxy for the performance of a nation, now it is spending on research and development (R&D). "More R&D" has been the cry of scientists and engineers for many decades. Economists, however, have long despaired at such naivety: they argue that invention and innovation are just one source of growth, and that both are themselves shaped by economic forces. It is simply not the case, they say, that the performance of national economies is determined by R&D spending. On the other hand, economists found that their own models of economic growth accounted for only a small fraction of actual growth, and many were tempted to attribute the 'residual' amount to tech-

nological progress. Nevertheless, historical cost-benefit analyses regularly showed that single technologies, such as steam engines in the Industrial Revolution or railways in the late-nineteenth-century United States, did not have the major economic impact loosely attributed to them. Coming to grips with the origins, nature and significance of technological change has proved to be remarkably difficult.

Over the years, the writings of Nathan Rosenberg on technological innovation have provided an invaluable guide to these often intractable issues. They have been rightly applauded not only for their rare lucidity but also for their freshness and suggestiveness. Rosenberg is a master of the well-crafted thought-provoking essay. Indeed, he is best known through two previous collections of his essays, *Perspectives on Technology* (1976) and *Inside the Black Box* (1982). This latest collection is very much in the same tradition and will undoubtedly be widely read. If you read nothing else on technological innovation, you will not go wrong by reading Rosenberg. One of the great virtues of his work is that it is easily accessible to the general reader. Furthermore, Rosenberg's enthusiasms and interests are good reflections of the interests of economists of technical change, and of other students of science and technology.

Rosenberg, often writing with a colleague, has also made a reputation as a quality controller of the literature on innovation. This volume contains an excellent example. In a paper with Claudio Frischtak he demolishes the view, widely canvassed in the early 1980s, that there have been long swings of economic activity powered by clusters of key technological innovations. They doubt the existence of these long waves, and then go on to argue that proponents of technological long waves did not put forward an adequate mechanism for the generation of long waves by innovations. They go on

to ask, however, what those mechanisms might be. Proponents of the long-wave view, they argue, simply did not recognize the complexities of the issues involved.

In common with many students of technological change, Rosenberg has been critical of the unrealistic, and often unhelpful, assumptions of neo-classical economists about technology. One way in which he has furthered this argument is by producing essays on past economic analysts of technological change. He wrote an important essay on Karl Marx and the economic role of science in the 1970s, and in this collection takes on the cases of two other much cited but often misunderstood students of technology: Babbage and Schumpeter.

Charles Babbage probably owes his contemporary fame to the ubiquity of the computer and the exaggerated role he has been given as its creator. But readers of Marx would know Babbage, along with the chemist Andrew Ure, from the footnotes to *Das Kapital*. Marx relied on their analyses of industrial technology and the division of labour in his chapter on modern industry. Rosenberg elegantly describes Babbage's innovative thinking on the benefits to employers of the division of labour and how Babbage's now famous calculating engines were intended to replace human 'computers'. His essay on the Austrian economist Joseph Schumpeter, who spent the last years of his professional life at Harvard, is also a model analysis of the works of a complex and rich thinker. Both pieces are excellent introductions to the key work of these seminal men.

Rosenberg stresses the centrality of uncertainty in technical change: in policy terms he argues for the need to follow many possible technical paths, the need for diversity in research and the need to plan for flexibility in future. He argues also for the importance of recognizing the significance of the path-dependence of large-scale technical change. Forecasting of the technological future, he shows, has been notoriously poor, and, perhaps most refreshingly of all, he studies technical change in industries ignored by high-technology enthusiasts; in this volume, large-scale chemical processing and the wood industry. His work stands as a warning, too often unheeded, to those who believe that they can know the technological future, and who would confine temporarily unfashionable industries to the dustbin of history. One of Rosenberg's great insights is to show that despite the futurist orientation of studies of technological innovation, there is a great deal to learn from the economic history of technology: after all, we have lived in technological societies for a very long time. □

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A SATIRICAL Egyptian papyrus (1196–945 BC) showing a lion and gazelle playing a board game. Such games were popular in ancient Egypt. One called 'senet' ('passing') had the most cultural and religious significance, and was often placed in tombs to provide a pastime for the deceased in the afterlife. Taken from *Reading Egyptian Art: A Hieroglyphic Guide to Ancient Egyptian Painting and Sculpture* by Richard H. Wilkinson. Newly published in paperback by Thames & Hudson, £9.95.

Sophistry and illusion

Frank J. Tipler

The Fire in the Equations: Science, Religion and the Search for God. By Kitty Ferguson. Bantam: 1994. Pp. 308. £16.99.

GOD is making an appearance in many popular science books these days. Leon Lederman, a Nobel prizewinner, called his recent book *The God Particle*, cosmologist George Smoot asserts that finding the fluctuations in the cosmic background radiation is like "seeing God" and Stephen Hawking tells us that the aim of science is knowledge of the "Mind of God".

Such statements seriously mislead the average person, who believes that scientists are finding the personal God of traditional theology. Nothing could be further from the truth. Lederman calls the Higgs boson the "God Particle" because it is the most important particle in particle physics today; Smoot means that, when contemplating the cosmic radiation, he experiences a feeling of awe analogous to that of religious believers; and Hawking's phrase is shorthand for the Theory of Everything. All three physicists — like most scientists of this century — describe themselves as agnostics or atheists. They do not believe in a Person who created the Universe.

Kitty Ferguson develops what I shall call a "sophisticated" God-of-the-Gaps theory. The old "naïve" God-of-the-Gaps theory found God in the gaps in our

knowledge: the early nineteenth-century naturalists invoked God to explain the adaptation of living organisms to their environment. The sophisticated version finds the gap in the foundations of science. The most basic physical laws, Ferguson claims, are so strange and their underpinnings so shaky that invoking God as the ultimate source is no less arbitrary. She provides a fairly good overview of many of the models of fundamental reality now being discussed by physicists, but I'm afraid her knowledge of these models is too shallow to permit a deep discussion of their possible philosophical relevance. For example, in my own area of expertise she correctly points out that the singularity theorems probably mean that the Universe had an infinite density a finite proper time in the past. Her inference: the Einstein equations broke down then, and therefore something more basic underlies them. But it could also mean that the Einstein equations are predicting a new form of 'stuff' — singularities, just as real as matter. Or it could mean that proper time is an inappropriate timescale in cosmology: in York time, a standard timescale in computer simulations of general relativity, the singularities are at temporal infinity. She does not adequately address David Hume's suggestion that the Universe itself may be logically necessary. This possibility underlies the search for a Theory of Everything.

Whatever the ultimate foundation of reality, if it is to be called "God" rather

than "the Universe", this ultimate level should be "personal" in some recognizable sense. Ferguson gives no reason whatsoever for believing it is, so her book is as misleading as the statements by Lederman *et al.* She is very hard on Hume for rejecting miracles and the possibility that God may actually be present in a mystical experience. Hume contended that the overwhelming observational evidence that the dead do not rise must be given due weight against claims of having seen Jesus after his death, the central miracle of Christianity. Ferguson asserts with the English physicist-theologian John Polkinghorne that Hume was "an intransigent sceptic who would never accept any evidence contradicting his prior expectation. . . . [this] is the antithesis of being open to the truth. It is certainly uncongenial to the habits of thought of a scientist If any witness could have convinced David Hume, it would have had to be David Hume."

I regard Hume as an open-minded sceptic. He knew that the human sensory system can easily misinterpret unfamiliar stimuli. So rationally he would probably not have believed his own 'observations' of a risen dead man. Timothy Ferris has adduced that the mystical experience of God's presence — a feeling of the unity of All — is due to a malfunction of a brain program responsible for integrating the mind into a single personality. But Hume, like a scientist, would have been more open to a reported miracle if there were first some consistent physical theory for a personal God who occasionally intervenes in His creation.

For scientists to take God-talk seriously, a book on science and religion would have to contain statements such as: "If God exists, then the top quark must have a mass of 185 ± 20 GeV; if God is a Person, then the Higgs boson must have a mass of 220 ± 20 GeV; and if She will one day raise us all to live forever in Heaven, then the temperature fluctuations $\Delta T/T$ of the cosmic background radiation must be less than 6×10^{-5} ".

Don't hold your breath. In a book suggesting God may be in the equations, there should at least be one equation. I can imagine what Hume would say about this book: "If we take in our hand any volume; of divinity or school metaphysics, for instance; let us ask, *Does it contain any abstract reasoning concerning quantity or number?* No. *Does it contain any experimental reasoning concerning matter of fact and existence?* No. Commit it then to the flames: for it can contain nothing but sophistry and illusion". I cannot agree with Hume on the flames. To burn it, you would have to buy it. □

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Carbon scaffolding: building acetylenic all-carbon and carbon-rich compounds

François Diederich

The preparation of acetylenic molecular and polymeric carbon allotropes and carbon-rich nanometre-sized structures opens new avenues of fundamental and technological research at the interface between chemistry and materials science. Unusual structures, high stability and useful electrical and nonlinear optical properties are some of the desirable characteristics of these materials.

At a rapidly developing frontier of synthetic chemistry, many researchers are aiming to prepare carbon allotropes¹ and carbon-rich compounds² with unusual structural, electronic and optical properties. Many of these materials are constructed from acetylene-based scaffolding³. This research has gained much encouragement from the explosive growth of fullerene research, following the preparation of bulk quantities of fullerenes in 1990 (ref. 4) and the subsequent preparation of many chemical derivatives⁵. Over the same period, there has been growing interest in the possibility that rigid acetylenic molecular rods of defined length could serve as molecular wires⁶ in molecular electronics applications⁷. Large conjugated carbon surfaces, different from graphite, could exhibit metallic character⁸ or, as a result of high polarizability, interesting third-order nonlinear optical properties⁹. Densely cross-linked all-carbon and carbon-rich crystalline solids could show exceptional thermal stability¹⁰, a high degree of hardness¹¹, and unusual mechanical properties such as strong shear deformation modes leading to a negative Poisson's ratio¹². Less dense materials could incorporate rationally designed molecular pores for recognition and catalysis in crystals and in the liquid phase².

Interest in acetylenic scaffolding dates back to the early days of acetylene chemistry, when Baeyer studied the oxidative Glaser coupling of acetylenes in the search for infinite linear chains of pure carbon¹³. A century later, the preparation and characterization of infinite linear polynes $[-(C\equiv C)_x-]$, named "carbynes", were actively pursued using a variety of methods^{14–16}. But reports of the preparation and structure of these materials, which are calculated to be one-dimensional conductors, remain controversial¹⁷. Materials closest to the infinite carbyne structure are the extended polynes reported in 1972 by Walton and co-workers¹⁸, which incorporate up to 16 conjugated $C\equiv C$ units and are stabilized by terminal triethylsilyl (Et_3Si) protecting groups. A few years later, the polyynylcyanides $H-(C\equiv C)_n-C\equiv N$ ($n=2, 3, 4$) were detected by Kroto and co-workers in the radio spectra of interstellar molecular clouds¹⁹. Gas phase carbon-cluster experiments at Rice University aimed at simulating the origin of the polyynylcyanides in red-giant (carbon) stars led in 1985 to the first experimental evidence for the formation of buckminsterfullerene, C_{60} , and initiated the subsequent vigorous world-wide fullerene research²⁰.

Macrocyclic acetylenic rings have also attracted considerable interest in the past. Following a theoretical proposal²¹, the groups of Sondheimer²², Staab²³ and Nakagawa²⁴ prepared in the 1960s and 1970s a variety of planar conjugated macrocyclic π -systems containing acetylenic bonds ('dehydroannulenes') and investigated whether these compounds obeyed the Hückel rule and showed aromatic ($(4n+2)$ π -electrons) or antiaromatic ($(4n)$ π -electrons) behaviour¹⁵. Other studies investigated the intramolecular reactivity of two triple bonds in cyclic diynes as a function of distance and orientation^{3,26}. Scott and co-workers synthesized a series of 'exploded cycloalkanes' in which

$-C\equiv C-$ ("n-pericyclynnes") or $-C\equiv C-C\equiv C-$ fragments are inserted between each pair of adjacent sp^3 -hybridized C atoms in n -membered cycloalkanes ($n=3-6$)^{27–29}. By photoelectron and electron absorption spectroscopy, they studied the extent of orbital interactions ('homoconjugation') between the acetylenic fragments that are separated by the 'insulating' sp^3 -C atoms which all bear either dimethyl or spirocyclopropyl groups.

Here I review the progress made in the synthesis of acetylenic all-carbon molecules and polymeric networks. A variety of practical (and impractical) structural proposals for two-dimensional and three-dimensional all-carbon networks that differ from graphite and diamond^{1,8,10,12,30–36} have been made over the past 20 years. Some of the precursors to acetylenic networks are perethynylated molecules such as perethynylated dehydroannulenes¹ (acetylenic conjugated macrocyclic π -electron systems with $-C\equiv CH$ groups at all peripheral valences) and cyclo[n]carbons (monocyclic n -membered rings with all- sp -hybridized C atoms³⁷). The cyclocarbons, in addition to linear polyyne fragments³⁸, also receive significant attention as potential intermediates in fullerene synthesis^{39,40}.

The polymerization of suitable carbon-network precursors is expected to encounter major obstacles in terms of stability, solubility and processibility of the increasingly large carbon-rich objects. Principles of supramolecular chemistry—using weak, non-covalent interactions to associate and spatially align molecular components in a defined way—may provide a means to control the orientation of precursors and intermediate oligomers in order to ensure high crystallinity of the intended extended networks. Finally, I review the unusual properties of stiff nanometre-sized carbon-rich molecular objects such as rods, surfaces and molecular pores, constructed by tetraethynylethene or phenylacetylene scaffolding. In the future, synthetic carbon allotropes and carbon-rich phases could take similar roles to diamond and graphite in technological applications; full exploitation of these promising new systems will rely on interdisciplinary approaches involving chemistry and material science.

All-carbon molecules

Cyclo[n]carbons (cyclo- C_n) are n -membered monocyclic rings of sp -hybridized C atoms with unique electronic structures resulting from two perpendicular systems of conjugated π -orbitals, one in-plane and one out-of-plane³⁷. Synthetic accessibility and stability considerations based on strain evaluation led to the selection of cyclo- C_{18} as the first preparative target. Calculations at various levels of theory made differing predictions for the electronic structure of this carbon molecule, which was expected to show special Hückel-aromatic stabilization as a result of the two orthogonal $(4n+2)$ π -electron systems⁴¹. Self-consistent field calculations with a 3-21G or larger basis set predicted that the cyclic acetylenic D_{9h} structure **1** (Fig. 1) with alternating bond lengths represents the ground-state geometry³⁷. But optimizations at the MP2 (Møller-Plesset second-order perturbation

theory) level including valence-electron correlations⁴² as well as density functional theory calculations⁴³, favour the cumulenic D_{18h} structure **2** (Fig. 1) as the most stable planar monocyclic structure.

Three synthetic routes to cyclo- C_{18} were explored; they pass through the direct synthetic precursors **3–5** (Fig. 1) which were all characterized by X-ray crystallography. Cyclo- C_{18} was first prepared in the gas phase by laser flash heating of **3**, and the neutral product was detected by resonant two-photon ionization time-of-flight mass spectrometry³⁷. The crystalline hexacobalt complex **4** (ref. 44) shows considerable bending of the three butadiyne units in the nearly planar C_{18} ring, with $C\equiv C-C$ angles as low as 161° . A comparison of the electronic absorption spectrum of the cyclocarbon complex to those of acyclic analogues strongly suggested the presence of macrocyclic π -conjugation in the central carbon ring. All attempts to free C_{18} from the co-ordinating metal atoms have failed so far.

The best route to the cyclocarbons cyclo- C_{18} , C_{24} and C_{30} originates from the carbon oxides **5–7** (Fig. 1)³⁹. These compounds are stable at room temperature but explode above $80^\circ C$; in addition, they are extremely sensitive to nucleophiles that induce polymerization through initial Michael addition. In Fourier-transform mass spectrometry (FTMS) experiments on **5–7**, the negative-ion mass spectra showed the expected successive loss of CO molecules from the precursor anions to give the cyclocarbon ions C_{18}^- , C_{24}^- and C_{30}^- (refs 39, 40). In the positive-ion mode, surprising gas-phase coalescence reactions of the cyclocarbon ions were observed with production of fullerene ions. Ion-molecule reactions starting from the cyclocarbon cations C_{18}^+ (formed by laser desorption of **5**) and C_{24}^+ (formed from **6**) led by way of distinct intermediates ($C_{18}^+ \rightarrow C_{36}^+ \rightarrow C_{54}^+ \rightarrow C_{72}^+ \rightarrow C_{70}^+ + C_2$ and $C_{24}^+ \rightarrow C_{48}^+ \rightarrow C_{72}^+ \rightarrow C_{70}^+ + C_2$, respectively) to fullerene C_{70} as the major product ion. Higher fullerene ions are also produced by further coalescence of C_{70}^+ with additional cyclocarbon molecules. These reactions are remarkably selective because formation of the C_{60}^+ ion is not observed. In contrast, the ion-molecule dimerization reaction of cyclo- C_{30} (produced from carbon oxide **7**) leads selectively to the buckminsterfullerene cation C_{60}^+ . No C_{70} is formed, and the extent of further coalescence reactions of C_{60}^+ leading to C_{90} or C_{120} is small.

The results of the FTMS studies with the carbon oxides **5–7** conclusively demonstrate that one reaction pathway to the formation of fullerenes is the coalescence of large cyclocarbon ions. In the synthesis of fullerenes by resistive heating of graphite under an inert cooling gas atmosphere, large cyclocarbon rings as well as polycyclic intermediates^{45,46} may form through coalescence of smaller linear polyyne fragments (C_n)⁴⁷. Evidence for the intermediacy of such reactive acetylenic fragments in the Krätschmer-Huffman process was obtained by evaporating graphite under He in the presence of dicyanogen (CN)₂ (ref. 38). Chromatography of the toluene-soluble product mixture no longer afforded fullerenes but rather a series of dicyanopolynes, $N\equiv C-(C\equiv C)_n-C\equiv N$ ($n=3-7$) formed on quenching of the linear polyyne intermediates. When H_2 is added to the inert gas, polynes $H-(C\equiv C)_n-H$ are obtained⁴⁸.

Strong support for the chain-to-ring-to-sphere mechanism for carbon growth was obtained in gas-phase ion chromatography experiments⁴⁵. Gas-phase ion chromatography can separate ions that have the same mass but which differ in isomeric structure or electronic configuration⁴⁹. These studies showed that large monocyclic, bicyclic and tricyclic rings are involved in fullerene growth, and that the collisional heating of these rings induces

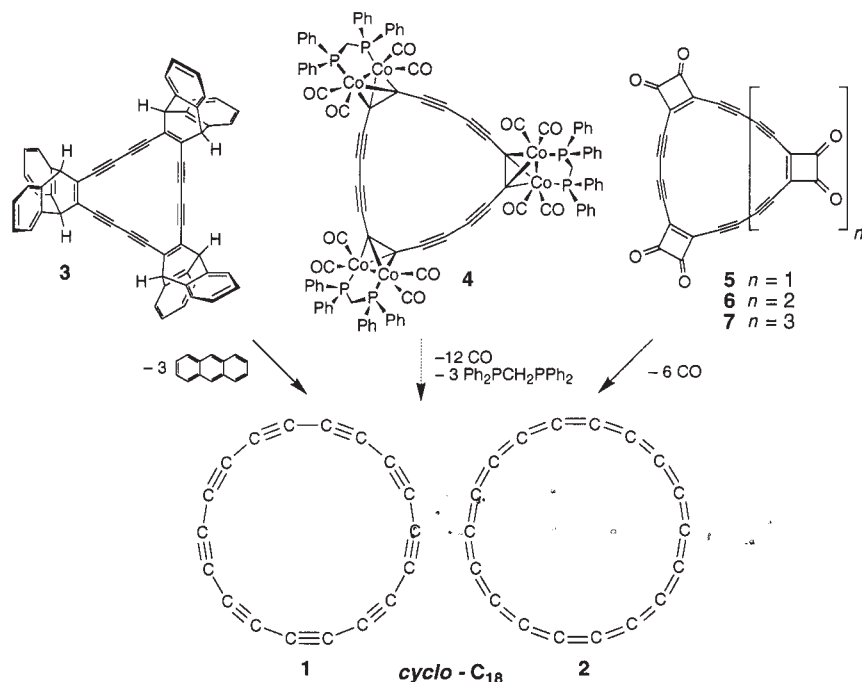


FIG. 1 Immediate precursors for the preparation of cyclo- C_{18} . The cyclocarbon was first prepared in the gas phase by laser flash heating of the hexadehydro[18]annulene **3**, which induced the stepwise elimination of three anthracene molecules in retro-Diels-Alder reactions. Both the D_{9h} -symmetrical acetylenic and the D_{18h} -symmetrical cumulenic structures of cyclo- C_{18} are shown.

isomerization to fullerenes accompanied by the loss of a small carbon fragment (for example, cyclic C_{40}^+ isomers $\rightarrow$ fullerene $C_{38}^+ + C_2$)⁵⁰. By the same technique, it was shown that laser vaporization of graphite generates C_{60}^+ ions consisting of the fullerene ion and a mixture of planar polycyclic polyyne ring isomers. The latter can be converted, through annealing, into the fullerene ion C_{60}^+ and, presumably, the large cyclocarbon cyclo- C_{60} (ref. 51).

Acetylenic-cumulenic carbon rings are substructures in a proposed new family of porous fullerenes, the 'fullereneynes'⁵². These cage molecules contain the same number of sp^2 -C atoms as either C_{60} or related larger fullerenes, but in addition, contain ≥ 60 sp -C atoms. In C_{120} (Fig. 2), compared to C_{60} , all pentagons remain intact but the former $C=C$ double bonds at the 6,6-ring junctions are now replaced by $C(sp^2)-C(sp)-C(sp)-C(sp^2)$ units of either cumulenic or acetylenic nature. The cages of the fullereneynes are porous and should allow formation of endohedral complexes, as well as shell complexes in which metal ions coordinate to the 12-membered acetylenic-cumulenic rings. Synthetic approaches to these interesting novel structures have not yet been reported. The heat of formation ΔH_f (g, C) of C_{120} , as calculated by the group increment method, is 24.4 kcal per g-atom C, which is more than double the value measured for C_{60} (ref. 53); it is therefore questionable whether fullereneynes like C_{120} could ever be isolated.

Carbon networks

Although a great variety of unnatural carbon networks can be devised, and many proposals have appeared in published work, until recently the synthesis of such networks has not received much attention from polymer and organic chemists. This may be explained by the fact that synthetic networks are thermodynamically less stable than graphite and diamond and were expected to undergo facile conversion into these natural allotropes. The current fullerene research has clearly shown that such expectations are not justified in all cases. The experimentally determined heat of formation per C atom for C_{60} is ΔH_f (g, C) = 10.16 kcal per g-atom C and for the more stable

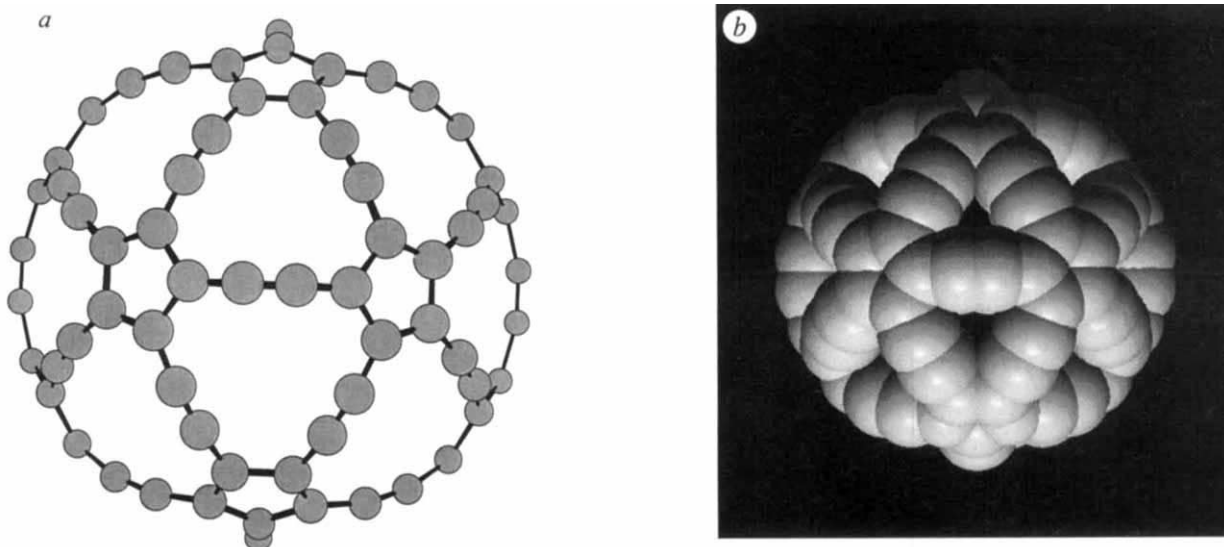


FIG. 2 Fullerene C₁₂₀ in a ball-and-stick representation (a) and as a space-filling model (b). For clarity, only one hemisphere of C₁₂₀ is shown.

higher fullerene C₇₀, 9.65 kcal per g-atom C (refs 54, 55). This compares with values of 0 kcal per g-atom C for graphite and 0.4 per g-atom C for diamond. Despite this large thermodynamic instability, the crystalline fullerenes are kinetically perfectly stable molecules even at high temperatures. By extrapolation, synthetic all-carbon networks for which no facile mechanisms for interconversion into graphite or diamond exist, should also exhibit high kinetic stability.

The choice of a target among the many imaginable all-carbon networks should be guided by the following criteria: (1) the network structures should neither be highly strained nor easily interconverted into graphite or diamond; (2) the new compounds should potentially exhibit interesting materials properties; (3) promising synthetic routes should be available; and (4) the preparation and characterization of monomers, dimers, and higher oligomers should allow extrapolation to the properties of the ultimate infinite-network targets.

In such a monomer-to-polymer approach, controlled oligotrimerization⁵⁶ of cyclo-C₁₈ or oxidative polymerization of hexaethynylbenzene **8** (ref. 57) could lead to the phenylbutadiyne network **9** (Fig. 3); oxidative polymerization of the elusive hexabutadiynylbenzene **10a** (C₃₀H₆, Fig. 3) would lead to a similar structure, but with larger pores which could serve as sites for a dopant or a guest component. Derivatives of **10a** are known: the preparation of hexasilyl-protected **10b** and the tert-butyl derivative **10c**, which was characterized by X-ray crystallography, was recently reported⁵⁸.

Highly strained cyclo-C₁₂ is a potential monomeric precursor to network **11** (Fig. 4) named graphyne, the most stable member of a large family of planar carbon sheets containing only *sp*- and *sp*²-C atoms⁹. According to semi-empirical MNDO (modified neglect of diatomic overlap) calculations, graphyne is expected to be planar within 0.01 Å. Valence effective hamiltonian (VEH) and extended Hückel theory (EHT) calculations predict **11** to be a large-bandgap semiconductor (*E*_g = 1.2 eV), and the network is expected to have interesting nonlinear optical properties including large third-order susceptibility. At 14.2 kcal per g-atom C, the calculated heat of formation Δ*H*_f(g, C) is only slightly higher than that of buckminsterfullerene; graphyne therefore should have sufficient stability for isolation and use as a new material.

The cross-conjugated carbon framework of tetraethynylethene **12a** (ref. 59) represents a repeat unit in a variety of two-dimensional networks including **15** (ref. 1) and **16** (Fig. 5). Therefore, the preparation of a specific network cannot be accomplished by simple oxidative polymerization of **12a**, but rather requires

a more characteristic macrocyclic precursor as starting material. Such a precursor to **15** is the perethynylated hexadehydro[18]annulene **13a** (Fig. 5), which was obtained by mild deprotection of the more stable trimethylsilyl-protected derivative **13b** with borax in methanol^{60,61}. Compound **13a** is highly unstable and can only be handled in dilute solution for short periods of time without decomposition. The electronic absorption spectra characterize the more stable silyl-protected derivatives **13b** and **13c** as Hückel-aromatic [18]annulenes with a large HOMO (highest occupied molecular orbital)–LUMO (lowest unoccupied molecular orbital) gap of 2.57 eV. The X-ray crystal structure analysis of **13b** (ref. 60) shows a perfectly planar carbon frame with linear butadiyne fragments in the 18-membered ring.

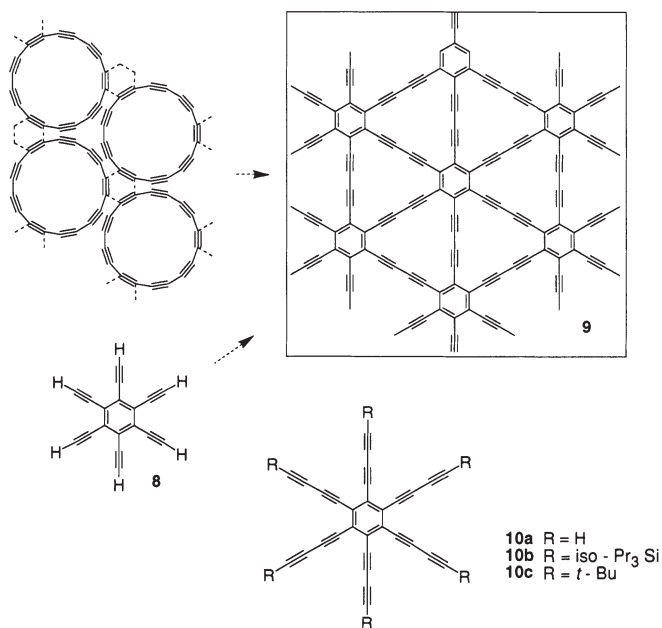


FIG. 3 Synthetic approaches to the planar carbon network **9**. Oxidative coupling of the elusive **10a** would give a network similar to **9** but with larger pores. Hexasilylated (**10b**) and peralkylated (**10c**) derivatives of **10a** are known⁵⁸.

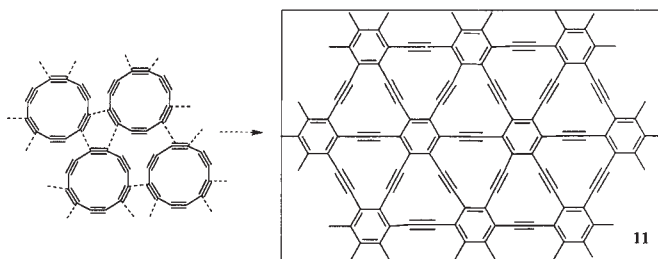


FIG. 4 Synthetic approach to graphyne (**11**) by oligotrimerization of cyclo-C₁₂.

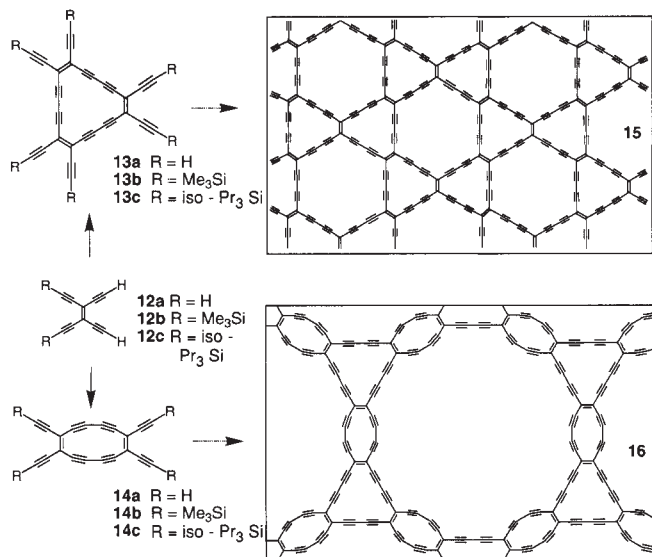


FIG. 5 Perethynylated antiaromatic [12] and aromatic [18]annulenes, derived from tetraethynylethene (**12a**) as macrocyclic precursors to the planar carbon networks **15** and **16**. The preparation of **13b**, **c** and **14b**, **c** was accomplished by oxidative coupling of **12b** or **12c**, respectively, under Hay coupling conditions (CuCl-TMEDA (*N,N,N',N'*-tetramethylethylenediamine), O₂, acetone).

Surprisingly, the oxidative cyclization of the *cis*-bisprotected tetraethynylethenes **12b** and **12c** (Fig. 5) yields not only the aromatic [18]annulenes **13b** and **13c** but also the strained antiaromatic [12]annulene derivatives **14b** and **14c** which are intermediates on the way to network **16**. The X-ray crystal structure of **14c** (ref. 61) shows a planar conjugated carbon frame and a strained 12-membered ring with significantly bent butadiyne fragments and C≡C—C angles as low as 164.5° (refs 61, 62). According to the electronic absorption spectra, **14b** and **14c** are antiaromatic [12]annulenes with a low HOMO–LUMO gap of 1.87 eV. Whereas the trimethylsilyl-protected derivative **14b** undergoes slow decomposition in dilute solutions even at –20 °C and cannot be isolated as a stable solid, dilute solutions of triisopropylsilyl-protected **14c** are much more stable and the crystalline compound only decomposes at 200 °C. Treatment of **14b** with borax in methanol affords solutions of unprotected **14a** as the highly unstable direct precursor to network **16**. Both experimental correlations and AM1 (Austin Model 1) calculations predict that oxidative polymerization of **14a**, with its relatively large external C≡C—C angles of 122–124°, should give exclusively the trimeric hexadehydro[18]annulene rings in **16** and no undesired dimeric tetrahydro[12]annulenes^{1,60,63}. An even more promising route to **16** proceeds through the silyl-protected C₆₀-sheet **17** and is currently under investigation (Fig. 6).

Whereas dilute solutions of **14c** are stable, concentration leads to rapid decomposition of the [12]annulene which only stops with the onset of crystallization. Crystals of **14c** remain unchanged for months when exposed to light and air at ambient temperature. This unusual stability results from the ‘insulating’ effect of the bulky triisopropylsilyl (TIPS) groups in the crystal and is not observed for the trimethylsilyl-analogue **14b**. Examination of the crystal lattice of **14c** shows (Fig. 7) that the delicate annulenic cycles are offset in stacking, so that they are completely surrounded by the bulky inert TIPS groups. The insulating ‘TIPS-effect’ was observed in several X-ray crystal structures of extended acetylenic compounds, and represents a general stabilizing mode for these compounds in the solid state^{60,64}.

Progress has been recently made towards the superdiamondoid network **20** (refs 1, 65 and Fig. 8). Tetraethynylmethane (**18**, Fig. 8) was elusive for many years^{66,67} until its synthesis was accomplished in 1993 by Feldman and co-workers⁶⁸. Solid **18**, like tetraethynylethene (**12a**), decomposes rapidly at room temperature in either the presence or absence of oxygen. A variety of other perethynylated organic molecules are currently being prepared and explored as precursors to crystalline carbon networks^{66,69–71}.

An entire class of three-dimensional networks (Fig. 9A) is structurally related to the metallic allotrope of carbon (**21**) initially proposed and studied computationally by Hoffmann *et al.*⁸ In this all-*sp*²-C-atom allotrope, two types of orthogonal polyacetylene chains that are connected by C—C single-bond hinges extend across the lattice. Unfortunately, there seems to exist no positive answer to the question “Can this metallic form of elemental carbon... be synthesized?” which was raised by Hoffmann *et al.* at the end of their communication⁸ on **21**. Later Elguero *et al.* made a proposal aimed at rendering this type of network more accessible³⁶ and modified the structure to **22** (Fig. 9A) by intercalation of an *sp*-C atom in the hinge between the two orthogonal chains of *sp*²-C atoms. Both **21** and **22** are very dense phases with calculated densities of 2.97 g cm^{–3} for **21** and 2.72 g cm^{–3} for **22**, compared to 3.51 g cm^{–3} for diamond and 2.27 g cm^{–3} for graphite. The two structures are essentially free of angle strain, but close nonbonded contacts (~2.5 Å interchain distances in **21** and ~2.56 Å distance between non-bonded allene units in **22**) presumably destabilize them to an extent which might prevent their isolation. To relieve the nonbonded strain and make this type of network synthetically accessible, Rubin and Diederich proposed¹ structure **23** (Fig. 9A) which resembles **22** in its allenic nature but contains butadiyne segments (—C≡C—C≡C—) instead of single bonds between the *sp*²-C atoms in the two orthogonal chains. Tetraethynylallene (**25**, Fig. 9B), a perethynylated [2]cumulene, should be a suitable precursor for **23**, but remains elusive; of the perethynylated [*n*]cumulenes, so far only the silyl-protected [3]cumulenes **26a** and **26b** (Fig. 9B) have been prepared⁷².

Unusual mechanical and thermal properties were predicted in computational studies by Baughman and Galvao¹² for network **24** (Fig. 9A), in which two orthogonal polydiacetylene chains —[C≡C—C≡C]_{*n*}— are connected by C—C single-bond hinges.

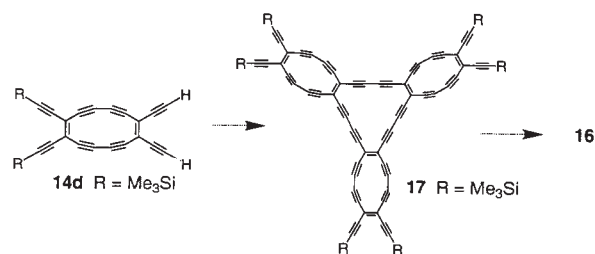


FIG. 6 Synthetic approach to the planar carbon network **16** by way of the silyl-protected planar C₆₀ molecule **17**.

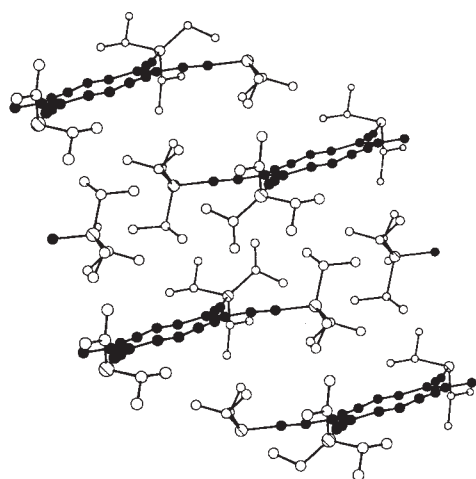


FIG. 7 Crystal lattice of the [12]annulene **14c**, showing the annulene carbon frame (black) surrounded by the triisopropylsilyl (TIPS) groups (only Si and C atoms shown in white).

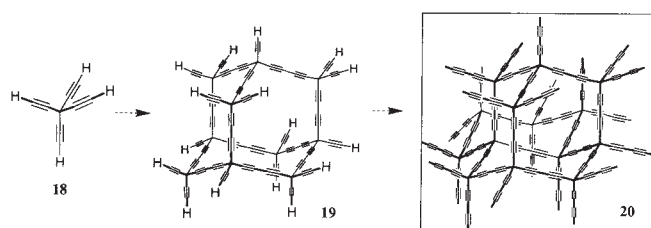


FIG. 8 The long-elusive tetraethynylmethane (**18**) has recently been prepared⁶⁸ and is a possible precursor *en route* to the 'superdiamond' network **20** by way of the expanded hexadecaethynyladamantane **19**.

This carbon phase is significantly less dense (1.789 g cm^{-3}) and less crowded than **21** with two orthogonal polyacetylene chains. In contrast to most materials that shrink laterally and become less dense when stretched, **24** is predicted to be a material that both expands laterally (that is has a negative Poisson's ratio) and densifies when stretched. Materials with a negative Poisson's ratio are called auxetics, and the predicted auxetic behaviour of **24** results from shear deformation modes about the C—C hinge bonds between the two orthogonal polydiacetylene chains. Other interesting material properties that have been predicted for **24** include negative thermal expansion, dopant-controlled porosity and low-temperature polymorphism.

With the nanometre-sized molecule **27** (Fig. 10), a "quantum dot analogue"¹² of the hinged phases **21–24** was recently reported by Moore and co-workers². Continued repeat of the structural motif in **27** (Fig. 10) would lead to a hypothetical three-dimensional network in which two orthogonal poly-*m*-phenylacetylene chains are coupled together by C—C≡C—C hinges. The spherical compounds **27** and **28** (Fig. 10) were designed to form porous organic crystals and, together with a variety of other linear and macrocyclic poly(phenylacetylene)-based nanoarchitectures, are accessible through an efficient repetitive construction methodology^{73,74}. This phenylacetylene scaffolding recently culminated in the preparation of a stiff phenylacetylene dendrimer with the molecular formula $\text{C}_{1398}\text{H}_{1278}$, the largest known hydrocarbon with an estimated diameter of 12.5 nm (ref. 75).

Experimental challenges and obstacles

Research on crystalline polymeric carbon networks faces some

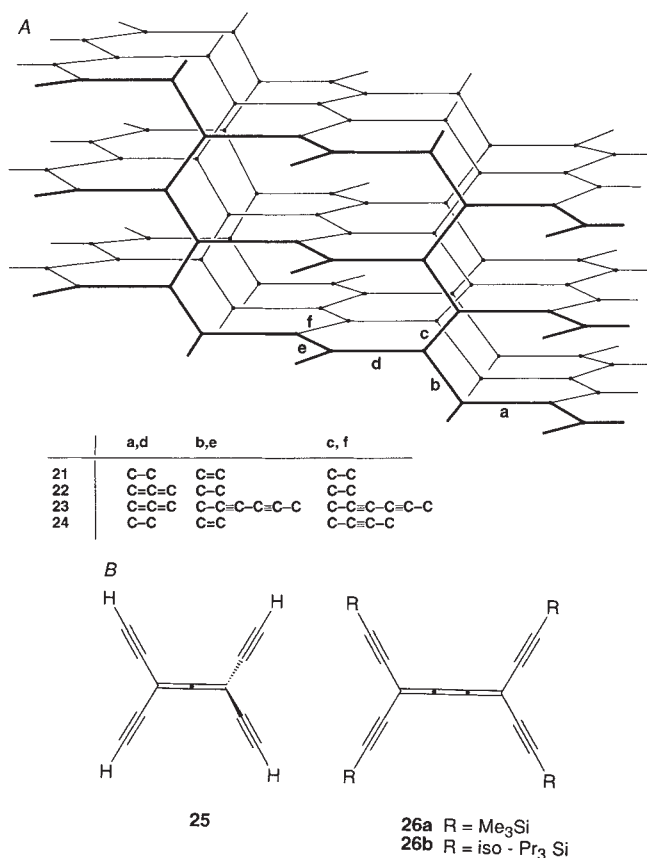


FIG. 9 A, Synthetic networks **21–24** with two orthogonal carbon chains connected by various hinges. B, Perethynylated [2]cumulene (tetraethynylallene) **25**, the precursor to network **23**, is still elusive whereas the silyl-protected perethynylated [3]cumulenes **26a, b** have recently been prepared.

formidable challenges beyond the selection and synthesis of stable suitable precursor molecules. The networks will become insoluble with increasing size and thus difficult to process or characterize. It might be impossible by conventional means to obtain a high degree of polymerization due to the likely insolubility of higher oligomers. Another problem could be a reduction in stability with increasing size of conjugated unsaturated networks, leading to intermolecular polymerization and to highly crosslinked systems with a low degree of crystallinity. To secure high crystallinity in production processes, growth from various initiation sites needs to be spatially controlled, and monomers or smaller oligomers forced to react in an oriented manner thereby preventing defects.

The stability and solubility of the polymerization precursors and carbon-rich sheets and rods prepared so far has surpassed expectations. Even compounds with six notoriously unstable terminal acetylenes such as **13a** can be handled in solution for periods of time and be brought to polymerization; similar behaviour can be expected for the unprotected compounds **17**, **31–33** (Fig. 11), and even **19**. The stability of molecules with terminal free acetylenes does not seem to decrease much with the size of the central carbon core to which these groups are attached: simple tetraethynylethene (**12a**) and the larger derivatives **13a** and **14a** show a similar stability. End-capping of peripheral valences is an obvious way to stabilize and solubilize large carbon molecular objects: compounds **31–33** discussed below are highly stable and readily soluble in hexane, toluene, or chloroform, and the same can be expected from much larger carbon surfaces. Interestingly, phenyl end-capping groups, in contrast to the bulky trialkylsilyl groups, significantly decrease the stability of

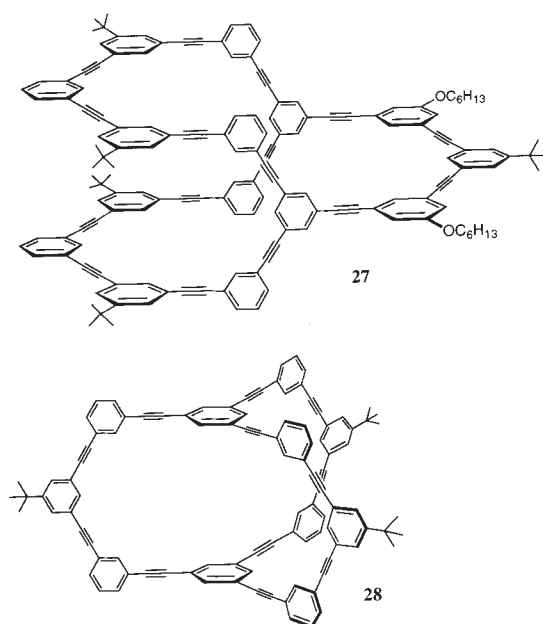


FIG. 10 Porous nanomaterials based on phenylacetylene scaffolding. Compound **27** is a 'quantum dot' from a network structurally related to the all-carbon phases **21–24** (Fig. 9A).

tetraethynylethene networks. They should be avoided because they stabilize intermediates of undesired anionic, cationic or radical polymerization side reactions.

Exploring the frontier of metastable compounds has fuelled much of the progress in modern chemistry, and the definition of what is possible has been continuously expanded as a result. In 1974, cyclobutadiene (C_4H_4) could only be isolated and studied in an argon matrix below 30 K; in 1994, the same molecule can be prepared and stored in air for weeks at ambient temperature, when incarcerated in a molecular container, a so-called carcerand^{76,77}. Principles of supramolecular chemistry, such as imprisonment, should also be useful in providing stability, solubility and processibility to polymeric carbon allotropes. As an example, large carbyne fragments $[-(C\equiv C)_n-]$ could be prepared through oxidation of smaller fragments

aligned in suitably sized channels of a zeolite. Alternatively, carbyne could be stabilized and rendered soluble by threading it into cyclodextrins, cyclophanes or other host macrocycles, thus generating a pseudorotaxane-type structure⁷⁸ (H. L. Anderson, personal communication).

Principles of supramolecular chemistry should be particularly useful in controlling the orientation of the monomers and the growing carbon objects, thus avoiding defects and ensuring high crystallinity in the ultimate extended networks⁷⁹. Experiments are under way to prepare network **9** by forming the reactive monomer cyclo- C_{18} through irradiation of carbon oxide **5**, oriented on the surface of a substrate like graphite (J. Frommer, N. S. Goroff and F.D., unpublished data). Such a procedure could allow the monitoring of polymerization with scanning tunnelling or atomic force microscopes. Alternatively, highly crystalline two-dimensional networks would be reached by oxidative electropolymerization of ultra-thin films of terminal acetylene monomers on electrode surfaces⁸⁰. Ultra-thin films of extended disk-shaped monomers such as **13a** could be generated by the Langmuir–Blodgett technique, and the polymerization could subsequently proceed in these ordered assemblies on liquid surfaces or absorbed on substrates^{81,82}. It has recently been shown that Langmuir–Blodgett film preparation does not require amphiphilic substrates, but that disk-shaped non-dipolar compounds such as phthalocyanines work as well⁸³. Another approach to orienting carbon-rich monomers for ordered polymerization would involve end-capping with diacetylenes, followed by topochemical polymerization in films or crystals to generate polydiacetylenes⁸⁴ with regularly attached carbon-rich monomers that subsequently react. The self-organization of end-capping long alkyl chains into layered structures could also provide suitable monomer orientation before polymerization⁷⁹.

Metal-templated self-assembly techniques⁸⁵ could dramatically facilitate the preparation of two- and three-dimensional crystalline acetylenic carbon networks which would subsequently be characterized by the rich variety of solid-state analytical methods⁸⁶. In the oxidative coupling of two acetylenes, a C–C bond of $\sim 130 \text{ kcal mol}^{-1}$ that joins two acetylenes ($C\equiv C-C\equiv C$) is formed irreversibly³⁷. Any error made in a conventional oxidative acetylene polymerization will therefore be irreversible, thus lowering the crystallinity of the resulting network. Judicious choice of a transition metal (M) and its ligands (L) should allow the reversible formation of σ -bis(acetylide) substructures $R-C\equiv C-ML_2-C\equiv C-R$, and thus the assembly of a metal-acetylenic network under thermodynamic

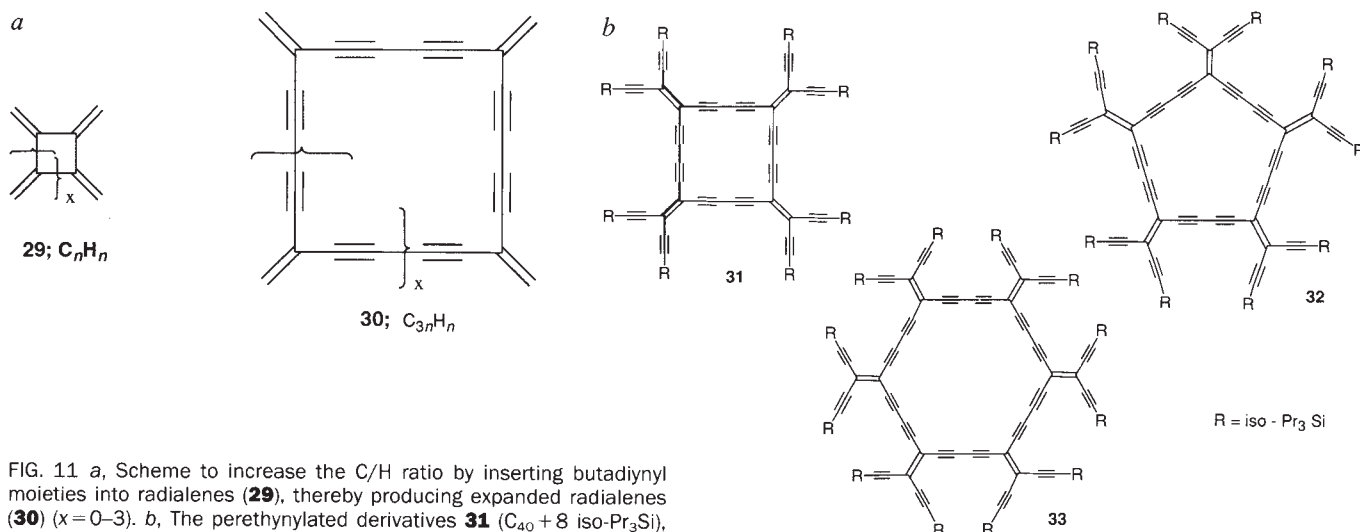


FIG. 11 a, Scheme to increase the C/H ratio by inserting butadiynyl moieties into radialenes (**29**), thereby producing expanded radialenes (**30**) ($x=0-3$). b, The perethynylated derivatives **31** ($C_{40} + 8 \text{ iso-Pr}_3\text{Si}$), **32** ($C_{50} + 10 \text{ iso-Pr}_3\text{Si}$) and **33** ($C_{60} + 12 \text{ iso-Pr}_3\text{Si}$) are the first known representatives of the expanded radialenes. Shown for **31** in bold is the longest linearly conjugated fragment in all three compounds. The

diameters of the carbon sheets (not including the $\text{iso-Pr}_3\text{Si}$ groups) are $\sim 17 \text{ Å}$ (**31**), $\sim 19 \text{ Å}$ (**32**) and $\sim 22 \text{ Å}$ (**33**).

control with error checking⁸⁷. In a second step, the all-carbon network would be generated by reductive elimination at the metal centre. While all of these fascinating techniques are being tested and introduced, the present-day methods of approaching the properties of the ultimate infinite-network targets by the preparation and study of monomers, dimers and higher oligomers will remain important.

Tetraethynylethene nanoarchitecture

Since the synthesis of tetraethynylethene (**12a**) in 1991 (ref. 59), a variety of differentially mono-, bis- and tris-protected derivatives have been prepared^{88,89}. This opened the way not only to the macrocyclic carbon network precursors **13** and **14** (Fig. 5) but also to novel carbon-rich nanoarchitecture with unusual structural and electronic properties such as the expanded radialenes **31–33** (ref. 90, Fig. 11b). These developments were only possible because the distance between the terminal *sp*-C atoms in the *cis*-enediynes substructure of tetraethynylethene derivatives is apparently too large for the Bergman cyclization to occur at ambient or mildly elevated temperatures^{91,92}. The Bergman cyclization is an important reaction in contemporary acetylene chemistry and produces highly reactive 1,4-dehydrobenzenes starting from *cis*-enediynes (*cis* $\text{RC}\equiv\text{C}-\text{R}'\text{C}=\text{CR}''-\text{C}\equiv\text{R}'''$); it also occurs in biotic systems and is responsible for the DNA-cleaving activity of the enediyne antitumour antibiotics like calicheamycin^{91,93}.

Radialenes (**29**) are a homologous series of all-*exo*-methylenecycloalkanes of molecular formula C_nH_n , ref. 94 (Fig. 11a). Upon insertion of butadiynyl moieties into the cyclic framework between each pair of vicinal *exo*-methylene units, the carbon-rich expanded radialenes **30** (C_{3n}H_n , Fig. 11a) are obtained. With the perethynylated derivatives **31–33**, the first representatives of the expanded radialenes were prepared starting from differentially protected tetraethynylethenes (Fig. 11b)⁹⁰. They possess large carbon cores with silyl-protected peripheral valences and can be viewed as $\text{C}_{40}+8$ iso- Pr_3Si (**31**), $\text{C}_{50}+10$ iso- Pr_3Si (**32**), and $\text{C}_{60}+12$ iso- Pr_3Si (**33**). Their high solubility and high stability (melting points above 260 °C) raises great hope that much larger carbon surfaces can be prepared and handled in the future, as long as the peripheral valences contain stabilizing and solubilizing groups such as the iso- Pr_3Si groups in **31–33**. A further expansion of the molecular dimensions should yield compounds which resemble large graphite or diamond fragments in that the carbon surfaces (rather than the heterofunctions at the peripheral valences) determine their material properties. At present, it is not known if such a transition occurs; testing the hypothesis of such a transition should be an important challenge for computational studies.

Laser desorption time-of-flight mass spectrometric techniques are particularly useful in the characterization of the expanded radialenes. The spectra, obtained in the negative-ion mode without matrix assistance, show strong molecular ions as well as a complete absence of any fragmentation or impurity peaks. Despite the high degree of unsaturation, **31–33** are only yellow-coloured and show similar end absorptions in the ultraviolet-visible spectra at ~500 nm. Apparently, only a reduced macrocyclic conjugation of similar magnitude is effective in all three compounds. Careful comparison showed that π -electron delocalization in **31–33** is limited to the longest linearly conjugated fragment (Fig. 11b) and that cross-conjugation, that is the conjugation between butadiynyl ($\text{C}\equiv\text{C}-\text{C}\equiv\text{C}$) fragments across the sp^2 -C atoms in the central ring, is not efficient. In view of the high stability of both expanded radialenes and fullerenes (which

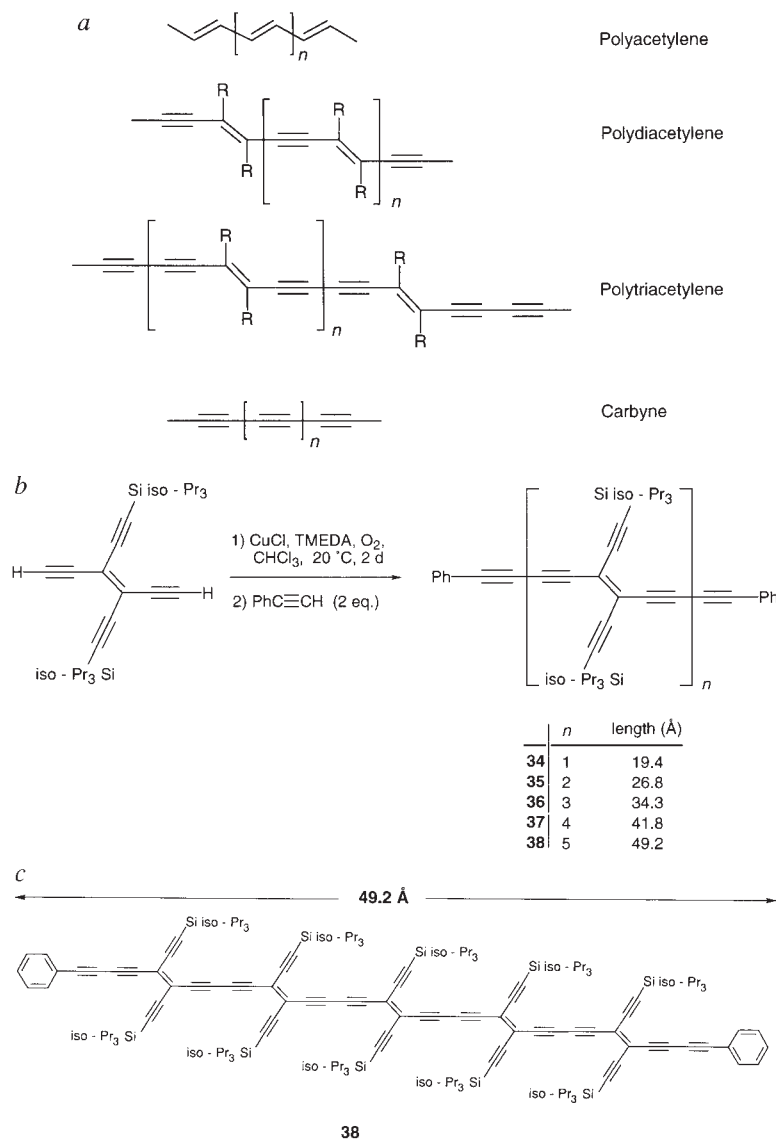


FIG. 12 a, Progression of polymeric backbones from polyacetylene to carbyne. b, Preparation of the soluble carbon rods **34–38** which have a polytriacetylene backbone. The length shown is the distance between the two extreme tips of the end-capping phenyl rings (TMEDA, see legend to Fig. 5). c, Representation of the longest carbon rod (**38**) isolated in pure form.

are also best described electronically as cross-conjugated molecules with [5]radialene substructures^{95,96}) it can be speculated whether the limitation of π -electron delocalization due to inefficient cross-conjugation is a general stabilizing principle of unsaturated carbon-based matter. If this is the case, such a principle could therefore serve as another useful guideline in the selection of all-carbon molecules and networks to be prepared. It should be noted that the electronic structure of graphite may also be viewed as cross-conjugated. Although cross-conjugation frequently occurs in organic matter, the understanding of the underlying orbital interactions and the extent of any resulting thermodynamic stabilization is insufficient and, for the benefit of future work on new carbon allotropes, should be improved^{97–99}.

By end-capping polymerization of *trans*-bis(triisopropylsilyl)-protected tetraethynylethene (Fig. 12b), a series of highly stable conjugated nanometre-sized molecular rods (**34–38**) was obtained⁶⁴. These oligomers possess the polytriacetylene backbone, which constitutes an intermediate step in the progression from polyacetylene¹⁰⁰, to polydiacetylene⁸⁴, to carbyne (Fig. 12a).

The carbon rods **34–38** (Fig. 12*b* and *c*), similar to the expanded radialenes **31–33**, are amazingly stable, high-melting-point materials that remain unchanged for months on the laboratory bench. X-ray crystal structures of monomeric **34** and dimeric **35** show nearly perfectly planar conjugated carbon frames including the phenyl rings. In the electronic absorption spectra of the highly coloured **34–38**, the wavelength of the end absorption increases with oligomeric chain length. A plot of these oligomeric end absorptions against the inverse of the oligomeric length allows extrapolation to the end absorption of an infinite polymer (that is, its solution bandgap), which is found to be $E_g = 2.3$ eV, comparable to the bandgap in many polydiacetylenes¹⁰¹. Preliminary investigations of the optical properties of a longer-chain polymer with a number-average molecular weight M_n of 6,900 (corresponding to a hexadecamer of ~12 nm length) confirmed this prediction⁶⁰. Electrochemical studies of **34–37** showed facile one-electron reductions of the oligomers, with the number of reversible one-electron reduction steps corresponding to the number of tetraethynylene moieties in each rod¹⁰². As the oligomer length increases, the first reduction occurs at increasingly less negative potentials ($E_{1/2}$ at -1.57 V *vs* ferrocene for **34** and -1.14 V for **37** in tetrahydrofuran). In contrast, none of the oligomers could be oxidized below $+1.0$ V *vs* ferrocene, which helps to explain their stability in air. The electrochemistry of **34–38** resembles that of the fullerenes, which also undergo facile reversible multiple one-electron reductions but are difficult to oxidize¹⁰³. This suggests a general property of unsaturated all-carbon materials.

The remarkable stability of oligomers such as **34–38** (or larger analogues) could make them useful as molecular wires in molecular electronics^{7,104,105}. To make contact with these 'wires', any desired substituent can be attached to the ends of the rods by changing the end-capping reagent. The lateral silyl groups can be easily exchanged for other substituents to allow attachment of such molecular wires to substrates such as silicon wafers.

Perspectives

The demonstration that fullerenes, despite their thermodynamic instability, are stable carbon molecules because of high kinetic stabilization has fuelled world-wide efforts to synthesize new molecular and polymeric carbon allotropes. There is now a justified belief that other forms of carbon can be prepared, and will not readily convert into the thermodynamically most stable natural forms, graphite and diamond. The new carbon allotropes are predicted to have exciting material properties and, if synthesized in bulk quantities, could play similar roles in technological applications as diamond and graphite (and, as expected in the near future, the fullerenes¹⁰⁶). Well designed approaches to the construction of new molecular and polymeric carbon allotropes become rewarding long before the ultimate targets are reached. The intermediate annulenes, expanded radialenes and molecular wires presented in this Review are novel carbon-rich materials with interesting and unusual structures and functions. In addition, they allow extrapolation to the properties of the ultimate all-carbon materials.

The success of attempts to construct new carbon phases is largely determined by high-quality organic synthesis. Modern acetylene chemistry, advanced by powerful new organometallic synthetic methodology, plays an important role in these efforts. The preparative challenges are formidable, and rival those in the more established synthesis of natural products and biologically active compounds. In addition, it is important to be receptive to unusual methods produced by other disciplines if unprecedented and unconventional synthetic methods (such as the bulk fullerene production process) are to be discovered¹⁰⁷. A profound understanding of how carbon atoms assemble to give carbon rods, mono- and poly-cyclic rings, and ultimately fullerenes in the Krätschmer–Huffman process, could help to unveil novel synthetic routes to new carbon allotropes.

Challenges in the synthesis of carbon allotropes are easily

matched by those in their analytical and material characterization. This research promises to be an active frontier in chemical sciences at the turn of the millennium. The ultimate targets, new carbon-based materials of technological interest, will only be reached by interdisciplinary approaches involving chemists and material scientists. Just as the barriers between biology and chemistry are currently collapsing¹⁰⁸, those between material science and chemistry will become increasingly permeable. □

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- Diederich, F. & Rubin, Y. *Angew. Chem. int. Edn engl.* **31**, 1101–1123 (1992).
- Wu, Z., Lee, S. & Moore, J. S. *J. Am. chem. Soc.* **114**, 8730–8732 (1992).
- Gleiter, R. & Kratz, D. *Angew. Chem. int. Edn engl.* **32**, 842–845 (1993).
- Krätschmer, W., Lamb, L. D., Fostiropoulos, K. & Huffman, D. R. *Nature* **347**, 354–358 (1990).
- Taylor, R. & Walton, D. R. M. *Nature* **363**, 685–693 (1993).
- Lehn, J.-M. *Angew. Chem. int. Edn engl.* **27**, 89–112 (1988).
- Carter, F. L. *Molecular Electronic Devices II* (Dekker, New York, 1987).
- Hoffmann, R., Hughbanks, R., Kertész, M. & Bird, P. H. *J. Am. chem. Soc.* **105**, 4831–4832 (1983).
- Baughman, R. H., Eckhardt, H. & Kertész, M. *J. chem. Phys.* **87**, 6687–6699 (1987).
- Karfunkel, H. R. & Dressler, T. *J. Am. chem. Soc.* **114**, 2285–2288 (1992).
- Neehan, T. X. et al. *Macromolecules* **21**, 3525–3528 (1988).
- Baughman, R. H. & Galvao, D. S. *Nature* **365**, 735–737 (1993).
- Baeyer, A. *Ber. dt. chem. Ges.* **18**, 674–681 (1885).
- Körshak, Y. V. et al. *Makromolec. Chem., rapid Commun.* **9**, 135–140 (1988).
- Kudryavtsev, Y. P. et al. *Carbon* **30**, 213–221 (1992).
- Whittaker, A. G. *Nature* **276**, 695–696 (1978).
- Smith, P. K. & Buseck, P. R. *Science* **216**, 984–986 (1982).
- Eastmond, R., Johnson, T. R. & Walton, D. R. M. *Tetrahedron* **28**, 4601–4616 (1972).
- Kroto, H. W. *Angew. Chem. int. Edn engl.* **31**, 111–129 (1992).
- Kroto, H. W., Heath, J. R., O'Brien, S. C., Curl, R. F. & Smalley, R. E. *Nature* **318**, 162–163 (1985).
- Sworski, T. *J. chem. Phys.* **16**, 550 (1948).
- Sondheimer, F. *Acct. Chem. Res.* **5**, 81–91 (1972).
- Staab, H. A., Ipaktschi, J. & Nissen, A. *Chem. Ber.* **104**, 1182–1186 (1971).
- Nakagawa, M. *Angew. Chem. int. Edn engl.* **18**, 202–214 (1979).
- Myers, A. G. & Finney, N. S. *J. Am. chem. Soc.* **114**, 10986–10987 (1992).
- Gleiter, R. *Angew. Chem. int. Edn engl.* **31**, 27–44 (1992).
- Houk, K. N. et al. *J. Am. chem. Soc.* **107**, 6556–6562 (1985).
- Scott, L. T., Cooney, M. J. & Johnels, D. J. *J. Am. chem. Soc.* **112**, 4054–4055 (1990).
- De Meijere, A. et al. *J. Am. chem. Soc.* **113**, 3935–3941 (1991).
- Balaban, A. T., Rentia, C. C. & Ciupitu, E. *Revue roum. Chim.* **13**, 231–247 (1968).
- Balaban, A. T. *Comput. Math. Applic.* **17**, 397–416 (1989).
- Stankevich, I. V., Nikerov, M. V., Bocharov, D. A. *Russ. Chem. Rev.* **53**, 640–655 (1984).
- Alberts, A. H. in *Integration of Fundamental Polymer Science and Technology 4* (eds Lemstra, P. J. & Kleintjens, L. A.) 396–401 (Elsevier, London, 1990).
- Merz, K. M. Jr, Hoffmann, R. & Balaban, A. T. *J. Am. chem. Soc.* **109**, 6742–6751 (1987).
- Otto, A. Z. *Chem.* **27**, 380 (1987).
- Eiguero, J., Foces-Foces, C. & Llamas-Saiz, A. L. *Bull. Soc. chim. Belg.* **101**, 795–799 (1992).
- Diederich, F. et al. *Science* **245**, 1088–1090 (1989).
- Grösser, T. & Hirsch, A. *Angew. Chem. int. Edn engl.* **32**, 1340–1342 (1993).
- Rubin, Y., Kahr, M., Knobler, C. B., Diederich, F. & Wilkins, C. L. *J. Am. chem. Soc.* **113**, 495–500 (1991).
- McElvany, S. W., Ross, M. M., Goroff, N. S. & Diederich, F. *Science* **259**, 1594–1596 (1993).
- Hoffmann, R. *Tetrahedron* **22**, 521–538 (1966).
- Parasuk, V., Amlöf, J. & Feyereisen, M. W. *J. Am. chem. Soc.* **113**, 1049–1050 (1991).
- Hutter, J., Lüthi, H. P. & Diederich, F. *J. Am. chem. Soc.* **116**, 750–756 (1994).
- Rubin, Y., Knobler, C. B. & Diederich, F. *J. Am. chem. Soc.* **112**, 4966–4968 (1990).
- Schwarz, H. *Angew. Chem. int. Edn engl.* **32**, 1412–1415 (1993).
- Taylor, R., Langley, G. J., Kroto, H. W. & Walton, D. R. M. *Nature* **366**, 728–731 (1993).
- Diederich, F. & Whetten, R. L. *Acc. Chem. Res.* **25**, 119–126 (1992).
- Heath, R. J. et al. *J. Am. chem. Soc.* **109**, 359–363 (1987).
- Bowers, M. T., Kemper, P. R., von Helden, G. & van Koppen, P. A. M. *Science* **260**, 1446–1451 (1993).
- von Helden, G., Gotts, N. G. & Bowers, M. T. *Nature* **363**, 60–63 (1993).
- Hunter, J., Fye, J. & Jarrold, M. F. *Science* **260**, 784–786 (1993).
- Baughman, R. H., Galvão, D. S., Cui, C., Wang, Y. & Tománek, D. *Chem. Phys. Lett.* **204**, 8–14 (1993).
- Beckhaus, H.-D., Rüchardt, C., Kas, M., Diederich, F. & Foote, C. S. *Angew. Chem. int. Edn engl.* **31**, 63–64 (1992).
- Beckhaus, H.-D. et al. *Angew. Chem.* (in the press).
- Kiyobayashi, T. & Sakiyama, M. *Fullerene Sci. Technol.* **1**(3), 269–273 (1993).
- Vollhardt, K. P. C. *Angew. Chem. int. Edn engl.* **23**, 539–556 (1984).
- Diercks, R., Armstrong, J. C., Boese, R. & Vollhardt, K. P. C. *Angew. Chem. int. Edn engl.* **25**, 268–269 (1986).
- Boese, R., Green, J. R., Mittendorf, J., Mohler, D. L. & Vollhardt, K. P. C. *Angew. Chem. int. Edn engl.* **31**, 1643–1645 (1992).
- Rubin, Y., Knobler, C. B. & Diederich, F. *Angew. Chem. int. Edn engl.* **30**, 698–700 (1991).
- Anthony, J. A. thesis, Univ. California at Los Angeles (1993).
- Anthony, J. A., Knobler, C. B. & Diederich, F. *Angew. Chem. int. Edn engl.* **32**, 406–409 (1993).
- Zhou, Q. & Swager, T. M. *Polym. Prepr., Am. chem. Soc. Div. Polym. Chem.* **34**, 193–194 (1993).
- Li, Y., Rubin, Y., Diederich, F. & Houk, K. N. *J. Am. chem. Soc.* **112**, 1618–1623 (1990).
- Anthony, J. A. et al. *Angew. Chem. int. Edn engl.* **33**, 763–766 (1994).
- Johnston, R. L. & Hoffmann, R. *J. Am. chem. Soc.* **111**, 810–819 (1989).
- Bunz, U., Vollhardt, K. P. C. & Ho, J. S. *Angew. Chem. int. Edn engl.* **31**, 1648–1651 (1992).

67. Alberts, A. H. & Wynberg, H. *J. chem. Soc., chem. Commun.* 748–749 (1988).
68. Feldman, K. S., Kraebel, C. M. & Parvez, M. *J. Am. chem. Soc.* **115**, 3846–3847 (1993).
69. Bunz, U. H. F. & Enkelmann, V. *Angew. Chem. int. Edn engl.* **32**, 1653–1655 (1993).
70. Neenan, T. X. & Whitesides, G. M. *J. org. Chem.* **53**, 2489–2496 (1988).
71. Stoner, T. C., Geib, S. J. & Hopkins, M. D. *Angew. Chem. int. Edn engl.* **32**, 406–409 (1993).
72. Van Loon, J.-D., Seiler, P. & Diederich, F. *Angew. Chem. int. Edn engl.* **32**, 1187–1189 (1993).
73. Zhang, J., Moore, J. S., Xu, Z. & Aguirre, R. A. *J. Am. chem. Soc.* **114**, 2273–2274 (1992).
74. Moore, J. S. & Zhang, J. *Angew. Chem. int. Edn engl.* **31**, 922–924 (1992).
75. Xu, Z. & Moore, J. S. *Angew. Chem. int. Edn engl.* **32**, 1354–1357 (1993).
76. Cram, D. J., Tanner, M. E. & Thomas, R. *Angew. Chem. int. Edn engl.* **30**, 1024–1027 (1991).
77. Cram, D. J. *Nature* **356**, 29–36 (1992).
78. Harada, A., Li, J. & Kamachi, M. *Nature* **364**, 516–518 (1993).
79. Stupp, S.-I., Son, S., Lin, H. C. & Li, L. S. *Science* **259**, 59–63 (1993).
80. Bauer, R. & Wendt, H. *J. electroanal. Chem.* **80**, 395–399 (1977).
81. Tachibana, H. & Matsumoto, M. *Adv. Mater.* **5**, 796–803 (1993).
82. Yang, H. C., Magnera, T. F., Lee, C., Bard, A. J. & Michl, J. *Langmuir* **8**, 2740–2746 (1992).
83. Souto, J., Tomilova, L., Aroca, R. & DeSaja, J. A. *Langmuir* **8**, 942–946 (1992).
84. Wegner, G. *Angew. Chem. int. Edn engl.* **20**, 361–381 (1981).
85. Whitesides, G. M., Mathias, J. P. & Seto, C. T. *Science* **254**, 1312–1319 (1991).
86. West, A. R. *Solid State Chemistry and its Applications* (Wiley, Chichester, 1989).
87. Diederich, F., Faust, R., Gramlich, V. & Seiler, P. *J. chem. Soc. chem. Commun.* (submitted).
88. Boldi, A. M., Anthony, J., Knobler, C. B. & Diederich, F. *Angew. Chem. int. Edn engl.* **31**, 1240–1242 (1992).
89. Hopf, H., Kreutzer, M. & Jones, P. G. *Chem. Ber.* **124**, 1471–1475 (1991).
90. Boldi, A. M. & Diederich, F. *Angew. Chem. int. Edn engl.* **33**, 482–485 (1994).
91. Nicolaou, K. C. & Dai, W.-M. *Angew. Chem. int. Edn engl.* **30**, 1387–1416 (1991).
92. Jones, R. R. & Bergman, R. G. *J. Am. chem. Soc.* **94**, 660–661 (1972).
93. Nicolaou, K. C. *Angew. Chem. int. Edn engl.* **32**, 1377–1385 (1993).
94. Hopf, H. & Maas, G. *Angew. Chem. int. Edn engl.* **31**, 931–954 (1992).
95. Hirsch, A., Soi, A. & Karfunkel, H. R. *Angew. Chem. int. Edn engl.* **31**, 766–768 (1992).
96. Taylor, R. J. *chem. Soc., Perkin Trans. 2* 3–4 (1992).
97. Pheilan, N. F. & Orchin, M. *J. chem. Educ.* **45**, 633–637 (1968).
98. Klein, J. *Tetrahedron* **39**, 2733–2759 (1983).
99. Traetteberg, M., Bakken, P., Almendinger, A., Lüttke, W. & Janssen, J. J. *molec. Struct.* **81**, 87–103 (1982).
100. Heeger, A. J. & McDiarmid, A. G. in *The Physics and Chemistry of Low Dimensional Solids* (ed. Alcacer, L.) 353–391 (Reidel, Dordrecht, 1980).
101. Baughman, R. H., Brédas, J. L., Chance, R. R., Eisenbaumer, R. L. & Shacklette, L. W. *Chem. Rev.* **82**, 209–222 (1982).
102. Schenk, R., Gregorius, H., Meerholz, K., Heinze, J. & Müllen, K. *J. Am. chem. Soc.* **113**, 2634–2647 (1991).
103. Xie, Q., Arias, F. & Echegoyen, L. *J. Am. chem. Soc.* **115**, 9818–9819 (1993).
104. Göpel, W. & Ziegler, Ch. (eds) *Nanostructures Based on Molecular Materials* (VCH, Weinheim, 1992).
105. Tour, J. M., Wu, R. & Schumm, J. S. *J. Am. chem. Soc.* **113**, 7064–7066 (1991).
106. Baum, R. M. *Chem. Eng. News* **71**(47), 8–18 (1993).
107. Nesper, R., Vogel, K. & Blöchl, P. E. *Angew. Chem. int. Edn engl.* **32**, 701–703 (1993).
108. Baum, R. M. *Chem. Eng. News* **71**(48), 50–51 (1993).

ACKNOWLEDGEMENTS. This work was supported by the Schweizerischen Nationalfonds zur Förderung der wissenschaftlichen Forschung, the US Office of Naval Research and the US National Science Foundation.

ARTICLES

Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*

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Complete enzymatic replication of DNA from the simian virus 40 origin has been reconstituted with T antigen and highly purified cellular proteins. DNA polymerase- α /primase functions primarily to synthesize RNA–DNA primers for initiation of DNA replication at the origin and for priming each Okazaki fragment. A polymerase switching mechanism requiring replication factor C and the proliferating cell nuclear antigen allows two molecules of DNA polymerase- δ to replicate both strands of the double helix conjointly.

THE description forty years ago of the double helical nature of DNA and the complementary array of base pairs suggested how DNA might replicate to produce two identical daughter molecules. Biochemical studies on the replication of either the bacterial genome or the DNA in bacterial plasmids and phages have elucidated the fundamental enzymology of DNA replication. In stark contrast, the process of eukaryotic cell chromosome replication is less well understood.

The development about ten years ago of a cell-free system for replication of DNA from the SV40, origin sequences (*ori*), initially using an extract from monkey cells¹ and subsequently using extracts from human cells^{2–4}, has greatly helped the identification of the eukaryotic cell DNA replication machinery. Biochemical fractionation of human cell extracts has resulted in the identification of the essential cellular replication components that combine with SV40 large T antigen to replicate SV40 *ori*-containing DNA. Recently, complete replication of SV40 *ori*-containing plasmid DNAs with highly purified proteins, including the two DNA polymerases α and δ , has been reconstituted⁵. This has provided a powerful system to investigate the mechanism of DNA replication and the role of each of the replication proteins.

Studies reported to date have suggested that the following events occur during bidirectional replication of DNA from the SV40 *ori* (reviewed in refs 6–10). A double hexamer of T antigen formed in the presence of ATP binds to the *ori* and causes structural distortion of the DNA¹¹. This stable initiator protein–DNA complex then binds the cellular replication protein A (RPA), a three-subunit single-stranded DNA-binding protein (reviewed in refs 6, 9). This allows more extensive unwinding of the DNA mediated by the DNA helicase function of SV40 T antigen (reviewed in ref. 10). The T antigen–RPA complex binds polymerase- α (pol α)/primase (refs 12–15 and reviewed in refs 6, 9) and a nascent RNA–DNA is synthesized at the *ori*^{16–19}. The cellular replication factor C (RFC) protein binds to the 3' end of the nascent DNA strand and loads proliferating cell nuclear antigen (PCNA) and DNA polymerase- δ (pol δ) on to the template, thereby replacing pol α ^{20–22}. The processive RFC/PCNA/pol δ complex then extends the nascent DNA strands to form the continuously synthesized leading-strand complex^{20,22,23}. Thus, initiation of leading-strand DNA replication requires a switch from pol α to pol δ ^{22,24–26}. The pol α /primase continues to prime and synthesize DNA discontinuously on the lagging-strand template.

A 5'-3' exonuclease (MF1) and DNA ligase I are required together with RNase H to complete DNA replication to yield covalently closed form-I product DNAs⁵. These proteins are required for removal of the primer RNA from Okazaki fragments and for DNA ligation to yield complete, lagging-strand DNAs (refs 27–29, and J. J. Turchi and R. A. Bambara, personal communication). We have exploited this reconstituted system to investigate further the mechanism of DNA replication. The results show that a DNA polymerase switching mechanism is required for the complete synthesis of every Okazaki fragment. Furthermore, our studies suggest that complete synthesis of both strands of DNA at a replication fork is a highly coordinated process.

Requirements for synthesis of DNA strands

In previous studies of SV40 DNA replication in the absence of MF1 and DNA ligase I, DNA replication occurred bidirectionally from the SV40 *ori* on both the leading-strand and lagging-strand templates, but the nascent Okazaki fragments remained unligated^{22,24}. This resulted in a multiply nicked, circular DNA product that migrated with form-II DNA marker in agarose gels run in the presence of ethidium bromide (Fig. 1a, lane 5). These replication products separated into a bimodal pattern after alkaline agarose gel electrophoresis (Fig. 1b, lane 5), with the short DNA products (average length, 0.6 kilobases (kb)) derived from the lagging strand (Okazaki fragments) and the longer (2.0 kb) products derived from the leading strand (refs 24, 30, and data not shown). When RFC, PCNA or pol δ was omitted from these reactions, also lacking MF1 and DNA ligase I/RNase H1, only the shorter lagging-strand DNAs were observed, demonstrating that they were synthesized by pol α and that RFC, PCNA and pol δ were essential for leading-strand replication (Fig. 1a and b, compare lane 5 with lanes 6–8; also see ref. 24).

When MF1 and DNA ligase I that contains trace amounts of RNase H were included in the complete replication reactions, covalently closed form-I DNA was produced (Fig. 1a, b, lanes 1, and ref. 5) but, unexpectedly the Okazaki fragments that were produced in the absence of RFC, PCNA or pol δ (Fig. 1b, lanes 6–8) were not ligated together, even in the presence of MF1/ligase I/RNase H1 (Fig. 1b, lanes 2–4). These results suggested that either leading-strand synthesis is required for completion and ligation of the Okazaki fragments, or that RFC, PCNA or pol δ (or a combination of these) is required to complete discontinuous, lagging-strand replication.

T4 holoenzyme and SV40 DNA replication

The human RFC, PCNA and pol δ proteins are functionally analogous to the bacteriophage T4 gp44/gp62, gp45 and gp43 replication proteins, respectively^{24,31}. We have shown that in the absence of MF1 and DNA ligase I/RNase H1, this phage T4 holoenzyme can replace the RFC/PCNA/pol δ holoenzyme so that the leading-strand template is replicated by the phage polymerase and the lagging-strand template is replicated by human pol α /primase²⁴. We therefore were curious as to whether or not the bacteriophage polymerase holoenzyme could replace the RFC, PCNA and pol δ proteins for lagging-strand synthesis and cooperate with human pol α to form completely replicated, form-I DNA products.

DNA replication reactions with SV40 *ori*-containing plasmid DNA, T antigen, pol α /primase, RPA, MF1/DNA ligase I/RNase H1 and topoisomerases I and II were supplemented with RFC, PCNA and pol δ (either partially purified RFC (fraction IV) or highly purified RFC (fraction VI) was used). As expected, covalently closed form-I DNA was detected by agarose gel electrophoresis in the presence of ethidium bromide (Fig. 2, lanes 2 and 3) and no form-I DNA product was observed in the absence of these three DNA replication factors (Fig. 2, lane 1). The phage T4 polymerase holoenzyme stimulated DNA replication (Fig. 2; compare lane 1 with lanes 4–6), but very little form-I DNA was produced (Fig. 2; compare lanes 2 and 3 with lanes

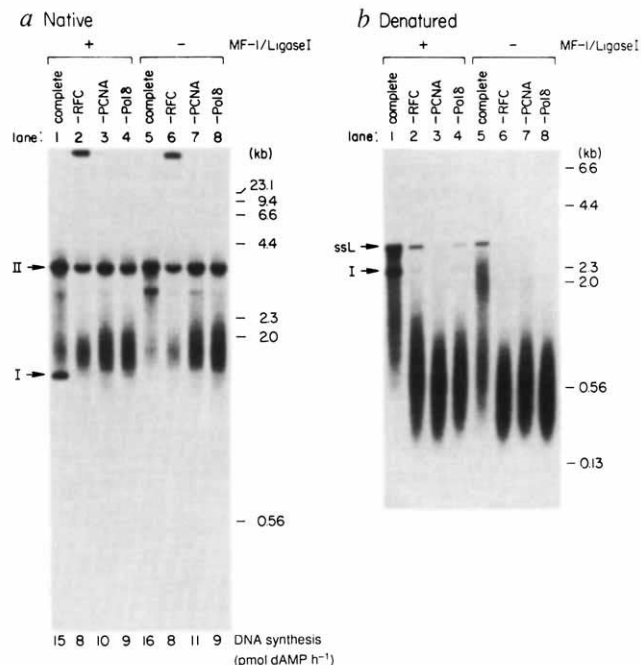


FIG. 1 Requirement for RFC, PCNA and pol δ for complete SV40 DNA replication. Reconstituted reactions of DNA replication from the SV40 *ori* were done with purified components in the presence (lanes 1–4) or absence (lanes 5–8) of MF1 and DNA ligase I/RNase H1. Either RFC (lanes 2 and 6), PCNA (lanes 3 and 7) or pol δ (lanes 4 and 8) was omitted from the complete reaction (lanes 1 and 5). Equivalent amounts of the purified products from each replication reaction were analysed by native agarose gel electrophoresis in the presence of ethidium bromide (a) and alkaline agarose gel electrophoresis (b). The amount of DNA synthesis for each reaction (equivalent to 50 μ l) is shown at the bottom of each lane in a. The positions of marker DNA are shown on the right. I, II and ssL denote covalently closed circular, relaxed DNA (form I), nicked circular DNA (form II) and single-stranded, full-length linear DNA, respectively. Note the DNAs in the wells (a) are catenated replicating intermediates because the DNA migrates faster than full-length single strands in a denaturing gel (b).

METHODS. Replication reactions were done with 6 μ g ml⁻¹ pSV011 DNA³⁰ under standard conditions^{3,5,22}. Components added in a 50- μ l reaction shown in lane 1 were 4 μ g T antigen, 2.5 μ g human RPA, 0.2 μ g bovine topoisomerase I, 90 ng bovine topoisomerase II, 0.4 μ g human PCNA, 1.8 μ g human RFC (fraction IV), 0.1 μ g human pol α /primase, 0.15 μ g bovine pol δ , 40 ng human MF1 and 0.5 μ g bovine DNA ligase I/RNase H1. Details of the purification procedures for T antigen, RPA, topoisomerases I and II⁴⁸, PCNA (K. Fien and B.S., unpublished and ref. 49), RFC⁵⁰, pol α /primase²¹, pol δ , MF1 and DNA ligase I⁵ have been described. The measurement of incorporated radioactivity and the analysis of products have been described^{5,22}.

4–6). Thus, although the phage T4 proteins could synthesize leading-strand DNA in the uncoupled reaction²⁴, they could not efficiently replace the human proteins for coordinated leading- and lagging-strand replication. It is possible that specific protein-protein interactions are involved in coordinating leading- and lagging-strand DNA replication and ligation of Okazaki fragments. This possibility was also suggested by the observation that human DNA ligase III could not substitute for DNA ligase I during complete SV40 DNA replication⁵.

Synthetic template for lagging-strand synthesis

The observation that RFC, PCNA and pol δ were required for complete replication of the lagging-strand in addition to being required for leading-strand DNA replication led to experiments designed to determine how both polymerases α and δ combine to complete Okazaki fragments. To help these studies, a synthetic lagging-strand template was designed (Fig. 3a). This lagging-

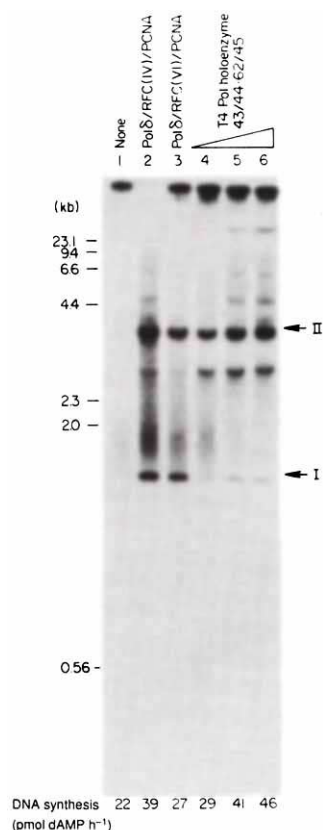


FIG. 2 Addition of bacteriophage T4 DNA polymerase holoenzyme to reconstituted SV40 DNA replication reactions. 'None' in lane 1 represents a reaction with T antigen, topoisomerases I and II, RPA, pol α /primase, MF1 and DNA ligase I/RNase H1. To this core reaction were added: lane 2, pol δ , RFC (fraction IV) and PCNA; lane 3, pol δ , RFC (fraction VI) and PCNA; lanes 4–6, various amounts of T4 DNA polymerase holoenzyme (gp43, gp44/62 and gp45). The reaction products were purified and subjected to native agarose gel electrophoresis in the presence of ethidium bromide. The amount of DNA synthesis for each reaction (50 μ l) is shown at the bottom of each lane. Replication reactions and components added into the reactions (50 μ l) were as described for Fig. 1 except that 60 ng RFC (fraction VI) in lane 3, 10 ng gp43, 0.5 μ g gp44/62 and 1 μ g gp45 in lane 6 were used. The ratio of the amounts of T4 holoenzyme used in lanes 4, 5 and 6 was 1:2:4, respectively.

strand model template contained a 445-nucleotide (nt) single-stranded DNA with an artificial Okazaki fragment containing a 15-nt RNA and a 227-nt labelled DNA annealed to the 3' end. A short 30-nt DNA fragment was annealed to the 5' end and represents a nascent Okazaki fragment. To assess complete lagging-strand synthesis, we measured the completion of DNA replication, removal of the RNA and ligation of one Okazaki fragment to the other by formation of a labelled 445-nt DNA product (Fig. 3b, c).

First, we examined whether RFC, PCNA and pol δ could support formation of ligated DNA product in concert with RPA and MF1/DNA ligase I/RNase H1. After the DNA synthesis reactions, the DNA products were separated by non-alkaline, denaturing polyacrylamide gel electrophoresis (Fig. 4). In the absence of added protein, the denatured DNA template reveals a 242-nt RNA–DNA molecule labelled U(+) (Fig. 4a, b, lane 2). The 15-nt RNA could be specifically removed by incubation of the template in alkali to reveal a 227-nt DNA molecule labelled U(–) (Fig. 4a, b, lane 1). Addition of MF1/DNA ligase I/RNase H1 also resulted in removal of the RNA (Fig. 4a, b, lanes 3). When RFC, PCNA and pol δ were added with RPA and MF1/DNA ligase I/RNase H1, a 445-nt fragment was detected, indicating that the 30-nt primer was elongated, the RNA was removed, and the two DNA molecules ligated together (Fig. 4a, lanes 5 and 7). The production of the ligated product depended on the addition of MF1/DNA ligase I/RNase H1 (Fig. 4a, lanes 4 and 6) and also on the presence of RPA (Fig. 4b, lanes 5 and 7), consistent with previous results using singly primed, single-stranded M13 DNA, showing that DNA synthesis by RFC, PCNA and pol δ requires RPA²³.

The formation of the 445-nt ligated product also depended on adding MF1 (Fig. 4c, lanes 2 and 3). In the presence of DNA ligase I, some removal of the RNA from the 242-nt RNA–DNA molecule was observed and was due to a small amount of RNase H activity in this protein preparation (Fig. 4c, lane 2). RNase H is required, together with the 5'–3' exonuclease activity of

MF1 for removal of the RNA in a similar synthetic template (J. Turchi and R. Bambara, personal communication).

The results in Fig. 4 show clearly that RFC, PCNA and pol δ are able, in the presence of RPA, to extend an Okazaki fragment. We next addressed the question of why pol α could not continue synthesis of an Okazaki fragment to produce ligated lagging strands during DNA replication. Addition of pol α /primase to reactions containing the synthetic lagging-strand template resulted in production of the 445-nt ligated product and this depended on the presence of MF1/DNA ligase I/RNase H1 (Fig. 5a, b, lanes 2–5). This product was obtained in the presence (Fig. 5a) and absence (Fig. 5b) of RPA, but RPA did inhibit the ability of pol α /primase to use this substrate (compare lanes 3 in Fig. 5a and b). Similar suppression of pol α /primase activity by RPA has been observed using poly(dA)/oligo(dT) as template-primer DNA²².

In marked contrast, the addition of RFC and PCNA, proteins known to recognize a primer template DNA in a structure-specific manner²¹, blocked the ability of pol α /primase to form the ligated product (Fig. 5a, b, lanes 6–9). Thus, RFC and PCNA prevent pol α from extending a DNA primer and completing an Okazaki fragment, and at the same time they help loading of pol δ so that this polymerase can complete Okazaki fragment synthesis.

DNA polymerase switching

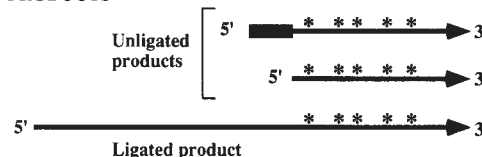
The reconstitution of DNA replication from the SV40 *ori* with highly purified components has enabled the functions of each replication protein to be studied. Using both a plasmid containing the SV40 *ori* and a model synthetic template for lagging-strand DNA replication by these purified proteins, the molecular anatomy of a eukaryotic cell DNA replication fork has begun to emerge. These studies also suggest a unique mechanism for lagging-strand DNA synthesis involving DNA polymerase switching.

Our current view of lagging-strand DNA synthesis is illustrated in Fig. 6. Lagging-strand DNA replication occurs by the discontinuous synthesis of RNA-primed Okazaki fragments. A

a TEMPLATE



b PRODUCTS



c SIZES

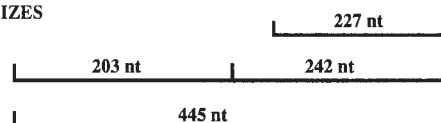


FIG. 3 Design of a model template for lagging-strand DNA replication. a, Structure of a synthetic, lagging-strand template. A 15-nt RNA linked to the end of a 227-nt ³²P-labelled DNA are shown as a shaded box and asterisks, respectively. A 30-nt DNA primer, representing a nascent Okazaki fragment, is also shown. b, Structures of the labelled DNA products formed during DNA synthesis of the lagging strand with the synthetic template. c, Length of each polynucleotide from the structures shown in a and b.

new Okazaki fragment is initiated by the assembly of pol α /primase onto the RPA-coated lagging-strand template (Fig. 6a, steps 1 and 2). The activity of pol α /primase on an RPA-coated, single-stranded template DNA is mediated by the primosome loading function of SV40 T antigen (refs 13, 15, 16, and reviewed in ref. 9). T antigen, which migrates in a 3' to 5' direction on the leading-strand template as a DNA helicase (reviewed in ref. 10), helps the synthesis of short RNA primers by its primase-loading function. The DNA polymerase activity of pol α /primase extends the RNA by synthesizing a short initiator DNA (iDNA). Evidence for the iDNA first emerged from studies of polyoma virus DNA replication³² and more recently, from studies of pharmacological inhibition of DNA polymerase activity during SV40 DNA replication *in vivo*³³. The length of the iDNA is currently estimated to be 34 nt and such short nascent DNA strands have been observed when antibodies that bind PCNA were added to block SV40 DNA replication in a crude cell extract¹⁷. In the purified DNA replication system, however, pol α /primase synthesizes RNA-DNA products considerably longer than this, suggesting that factors present in the crude extracts other than RPA, PCNA and RFC modulate the activity of pol α .

Once the iDNA is synthesized, the ATP-dependent, structure-specific DNA-binding protein RFC could bind to the 3' end of the iDNA and help the loading of PCNA and then pol δ (Fig. 6a, steps 3 and 4). The pol α /primase would be displaced from the template DNA and thus be prevented from completing DNA synthesis up to the previously synthesized Okazaki fragment. The biochemical functions of RFC and PCNA are consistent with this model^{20,21,31}. We propose that a DNA polymerase

switching mechanism occurs during the synthesis of every Okazaki fragment on the lagging-strand template in much the same way as a switch from polymerase α to polymerase δ occurs during initiation of leading-strand DNA replication at the SV40 *ori*²⁴.

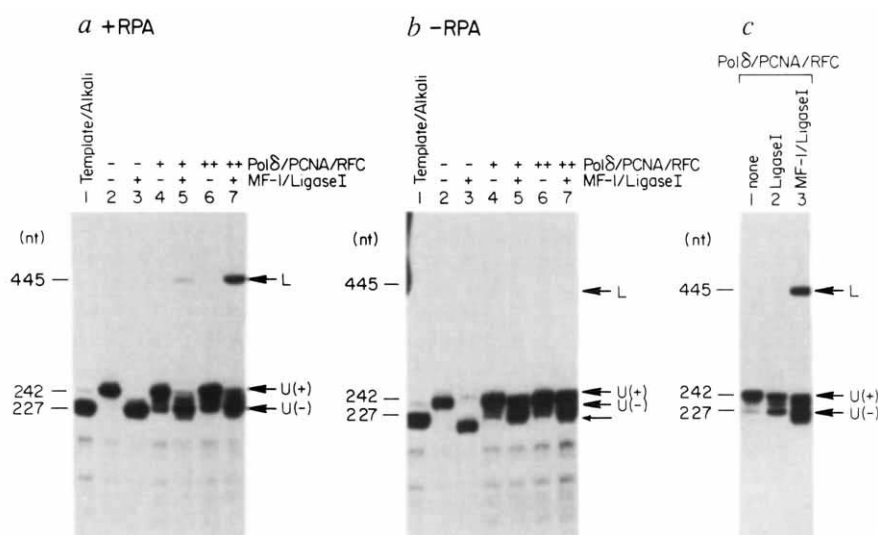
Completion of Okazaki fragment synthesis involves the synthesis of DNA by the RFC/PCNA/pol δ holoenzyme, removal of the RNA by RNase H and the 5'-3' exonuclease activity of MF1 and ligation of the DNA by DNA ligase I (Fig. 6a, steps 5-7). There is evidence (J. Turchi and R. Bambara, personal communication) for a cooperative role for RNase H and MF1 5'-3' exonuclease in removal of the RNA and this is consistent with other data^{27,28}. Aspects of this model are also consistent with the ability of the RFC/PCNA/pol δ holoenzyme complex to fill in a gap in a gapped synthetic DNA template^{29,34}. RFC, PCNA and RPA all contribute significantly to the polymerase specificity in lagging-strand synthesis because they cooperate to prevent pol α from completing Okazaki fragment synthesis and assist pol δ in adopting its role as the major replicative DNA polymerase.

Coordinated replication of both DNA strands

Early studies following fractionation of SV40 DNA replication extract suggested that PCNA was required for coordinated replication of both the leading and lagging strands³⁰. The current data extend this initial finding by demonstrating that RFC, PCNA and pol δ are all required both for leading- and lagging-strand DNA replication. Furthermore, previous experiments with the bacteriophage T4 holoenzyme have demonstrated that the phage polymerase complex can substitute for the pol δ com-

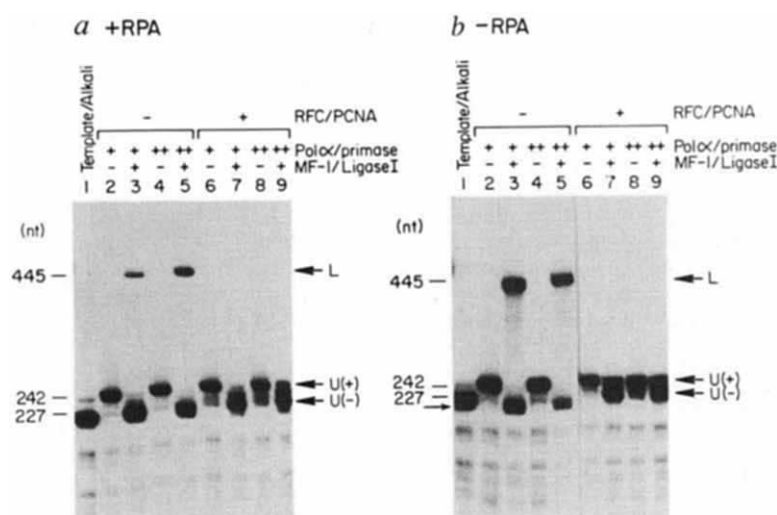
FIG. 4 Analysis of the products from DNA synthesis reactions by pol δ using the synthetic, lagging-strand template. The product from each reaction was purified and analysed by 8M urea, 5% polyacrylamide gel electrophoresis. Reactions were in the presence (a) or absence (b) of RPA. In a and b, components added were: lane 2, no component; lane 3, MF1 and DNA ligase I/RNase H1; lanes 4 and 6, pol δ , PCNA and RFC; lanes 5 and 7, pol δ , PCNA, RFC, MF1 and DNA ligase I/RNase H1. The concentration of either pol δ , PCNA or RFC used in lanes 6 and 7 was twice that used in lanes 4 and 5. Lane 1 shows the synthetic template after alkaline hydrolysis. c, Requirement for MF1 for the formation of ligated DNA product. DNA synthesis reactions with the synthetic template, including RPA, RFC, PCNA and pol δ , were in the presence of neither MF1 nor DNA ligase I/RNase H1 (lane 1), only DNA ligase I/RNase H1 (lane 2), and both MF1 and DNA ligase I/RNase H1 (lane 3). The position of marker DNA is shown on the left of each gel. L, U(+) and U(-) denote ligated product (445 nt), unligated DNA with the 15-nt-long RNA (242 nt) and unligated DNA without the 15-nt-long RNA (227 nt), respectively. The small arrow in b indicates the band migrating faster than the 227-nt-long unligated DNA in lane 1, suggesting digestion of DNA by MF1 beyond the RNA-DNA boundary in the absence of RPA (S.W. and B.S., unpublished results).

METHODS. The synthetic, lagging-strand template was constructed as follows: a non-labelled 15-nt RNA was synthesized in the T3 RNA polymerase reaction with pBluescript SK(-) DNA (Stratagene) which had been linearized and blunt-ended by *SacI* and T4 DNA polymerase. This 15-nt-long RNA was purified by 20% polyacrylamide gel electrophoresis and annealed to 4 μ g single-stranded, circular pBluescript SK(-) DNA. DNA synthesis from 3'-end of the RNA was then done with 100 μ Ci [α -³²P]dATP (800 Ci mmol⁻¹; Amersham), 250 μ M each dCTP, dGTP and TTP, and 500 units RNaseH(-) reverse transcriptase (BRL) in the buffer supplied with the enzyme. This reaction mixture was incubated at room temperature for 10 min and then at 37 °C for 60 min, followed by additional incubation for 30 min after adding 250 μ M cold dATP. Partial-duplex DNA containing the RNA segment was then purified and



annealed with a 40-mer oligodeoxynucleotide, which was complementary to the sequence of the pBluescript SK(-) DNA minus strand from nt 948-987, and finally the synthetic template was digested with *PvuII* and purified by 3.5% PAGE. Two *PvuII* sites exist on pBluescript SK(-) DNA; one of them is at nucleotide position 977 within the 40-mer oligodeoxynucleotide used above, and the other site is at nucleotide position 532, downstream from the annealed 15-nt RNA. DNA synthesis reactions with the synthetic template used ~0.1 μ g ml⁻¹ template DNA in the SV40 DNA replication reaction buffer³ supplemented with 100 μ g ml⁻¹ BSA, except an ATP-regeneration system was omitted. The reaction mixture was incubated at 37 °C for 60 min. The amount of each component used in a 50- μ l reaction was 0.1 μ g RPA (a and c), 25 ng (lanes 4 and 5) or 50 ng (lanes 6 and 7, and c) of pol δ , 0.32 μ g (lanes 4 and 5) or 0.63 μ g (lanes 6 and 7) RFC (fraction IV), 23 ng RFC (fraction VI) (c), 70 ng (lanes 4 and 5) or 140 ng (lanes 6 and 7, and c) PCNA, 8 ng (a and b) or 11 ng (c) MF1, and 0.1 μ g DNA ligase I/RNase H1.

FIG. 5 Analysis of the products from DNA synthesis reactions by pol α /primase using the synthetic, lagging-strand template. Reactions were in the presence (a) or absence (b) of RPA. The reaction conditions and product analysis were as described in Fig. 4. In a and b, components added were: lanes 2 and 4, pol α /primase; lanes 3 and 5, pol α /primase, MF1 and DNA ligase I/RNase H1; lanes 6–9, same as lanes 2–5 except RFC and PCNA were included. Lane 1 is the synthetic template subjected to alkaline hydrolysis. The amount of each component used in a 50- μ l reaction was as follows; 0.1 μ g RPA, 30 ng (lanes 2, 3, 6 and 7) or 60 ng (lanes 4, 5, 8 and 9) pol α /primase, 11 ng MF1, 0.1 μ g DNA ligase I/RNase H1, 0.63 μ g RFC (fraction IV), and 140 ng PCNA. L, U(+) and U(-) were as described in Fig. 4. A small arrow in B indicates the bands in lanes 3 and 5 migrating faster than a 227-nt-long band in lane 1 as described in Fig. 4.



plex during synthesis of the leading-strand template²⁴. In contrast, the phage T4 holoenzyme complex could not substitute for the pol δ complex to produce covalently closed, circular DNA products during DNA replication from the SV40 *ori* with T antigen, RPA, pol α /primase, MF1/ligase I/RNase H1 and DNA topoisomerases I and II. These data argue that specific protein-protein interactions between strand-specific polymerase complexes are important in coordinating synthesis of both strands at a replication fork. Similarly, a specific requirement for DNA ligase I from mammalian cells during SV40 DNA replication *in vitro*⁵ further suggests that specific protein-protein interactions are required to complete lagging-strand synthesis.

For these reasons, we propose that replication of the leading and lagging strands by pol δ are coordinated, perhaps as shown in Fig. 6b. In this model, the lagging strand is looped around so that the lagging- and leading-strand polymerase complexes can move along the DNA in concert, consistent with the model for a dimeric DNA polymerase at the *Escherichia coli* or phage T4 replication forks (reviewed in refs 35, 36).

Replication of both the *E. coli* chromosome and the bacteriophage T4 genome requires the same core DNA polymerase enzyme to replicate the leading and lagging strand (reviewed in refs 36–39). Thus, the apparent requirement for two DNA polymerase- δ holoenzymes for synthesis of both strands at a

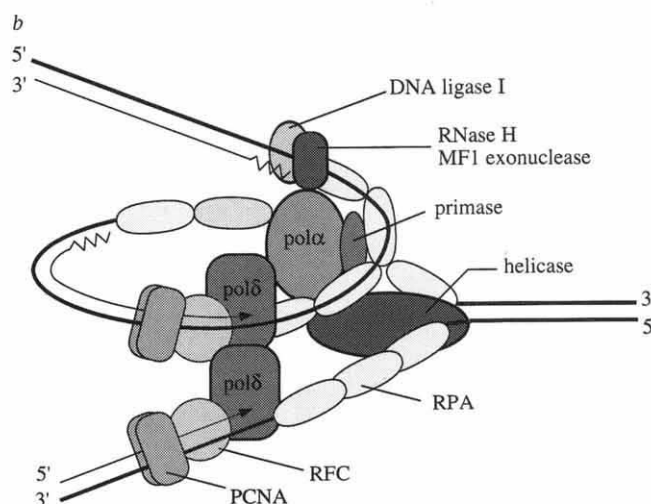
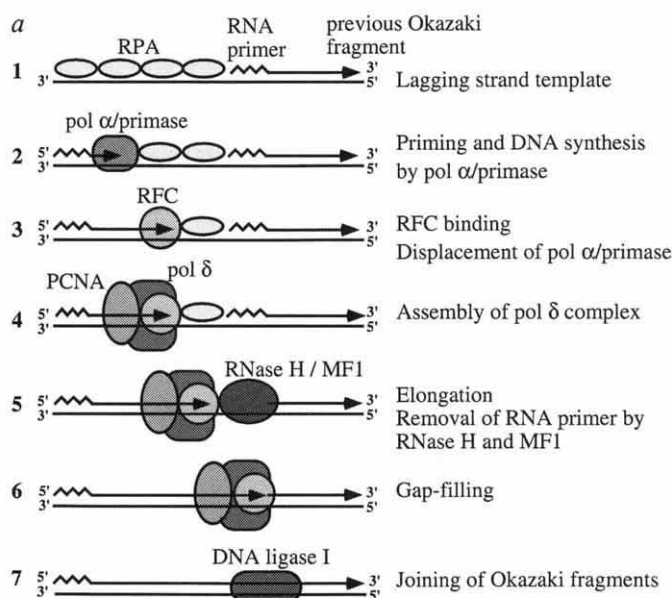


FIG. 6 Mechanism of lagging-strand synthesis at a eukaryotic cell DNA replication fork. a, Model for a DNA polymerase switching mechanism during lagging-strand replication (see text for details). b, Model for a multi-protein complex at a eukaryotic cell DNA replication fork, including one molecule of polymerase- α /primase complex which is about to synthesize an initiator RNA-DNA (see text) on the lagging-strand template

and two molecules of polymerase- δ ; one of them on the lagging-strand template is elongating the DNA strand from the initiator DNA previously synthesized by polymerase- α /primase, and the other on the leading-strand template is continuously replicating the leading strand. During SV40 DNA replication, the DNA helicase at the fork is SV40 T antigen.

eukaryotic cell DNA replication fork is analogous to the prokaryotic replication machinery. But here the similarity ends. At a eukaryotic replication fork, two molecules of pol δ and one molecule of pol α cooperate to synthesize DNA. The pol α is constitutively associated with DNA primase and its primary role is apparently to provide an initiator DNA for subsequent DNA synthesis by pol δ . In contrast, in the prokaryote systems, the primase is not constitutively associated with the DNA polymerase, but appears to prime DNA synthesis by cycling on and off the lagging-strand template (reviewed in ref. 38). There does, however, appear to be a polymerase switching mechanism during complete lagging-strand replication in *E. coli*. DNA polymerase I, rather than the principal replicative DNA polymerase, DNA polymerase III holoenzyme, is thought to be required during lagging-strand replication for gap-filling DNA synthesis and removal of the RNA primer⁴⁰. This aspect of DNA replication also seems to be different in eukaryotes because the principal replicative polymerase, pol δ , does not appear to use an RNA primer, but replicates the Okazaki fragment from a DNA primer (iDNA) made by pol α /primase, and then progresses to complete the gap-filling DNA synthesis. The 5'-3' exonuclease activity of MF1 may be analogous to the exonuclease activity associated with the RNA removal function of *E. coli* DNA polymerase I. It therefore appears that the prokaryotic and eukaryotic DNA replication machineries both use similar enzymatic activities but that these components are organized differently. This may be due to the fact that the eukaryotic DNA replication machinery has to cope with replicating DNA in a highly complex but organized chromatin template that is for the most part absent in prokaryotes, thereby necessitating mechanistic differences in replicating DNA. Alternatively, the coordinated replication of many replicons in a single cell might demand that the primase be controlled differently in eukaryotes.

The simultaneous synthesis of leading- and lagging-strand DNA by the polymerase δ holoenzyme would also position a

proof-reading exonuclease on both sides of the replication fork. The polymerase- δ subunit, unlike the polymerase- α catalytic subunit, contains a 3'-5' exonuclease activity which could ensure accurate DNA synthesis on both strands (reviewed in ref. 41). Indeed, proof-reading appears to be equally efficient on both the leading and lagging strands during replication from the SV40 *ori*⁴², unlike the situation that occurs during plasmid replication in *E. coli*⁴³.

Genetic investigations in the yeast *Saccharomyces cerevisiae* have demonstrated that another DNA polymerase, DNA polymerase- ϵ , is essential for cell viability and for completion of S phase⁴⁴. Using artificial DNA templates, others have implied that pol ϵ may be required for lagging-strand DNA replication^{34,45}. Despite the fact that DNA polymerase- ϵ was present in the crude cell extract from human cells that supported SV40 DNA replication, this enzyme was not purified as an essential protein for replication from the SV40 *ori*. DNA polymerase- ϵ is required for DNA repair both in human⁴⁶ and yeast⁴⁷ cell extracts and it may well be that this repair process is essential for cell-cycle progression and cell viability. Alternatively, it is possible that pol ϵ is dispensable for replication of the relatively short SV40 replicon but that it is required to replicate the relatively long replicons present in eukaryotic chromosomes. Finally, although our biochemical studies suggest that pol δ functions on both sides of the replication fork, it is possible that pol ϵ plays some role *in vivo* in DNA replication that has not been reproduced in the SV40 cell-free system.

Characterization of the SV40 DNA replication reaction has provided a solid foundation for future studies on the mechanism and regulation of chromosomal DNA replication in eukaryotes. In this system, however, SV40 T antigen functions as an initiator, DNA helicase and primosome-loading protein. It will be necessary, therefore, to investigate the biochemistry of DNA replication from cellular origins of DNA replication to achieve a full appreciation of eukaryotic cell DNA replication. □

Received 2 December 1993; accepted 10 February 1994.

- Li, J. J. & Kelly, T. J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6973-6977 (1984).
- Li, J. J. & Kelly, T. J. *Molec. cell. Biol.* **5**, 1238-1246 (1985).
- Stillman, B. W. & Gluzman, Y. *Molec. cell. Biol.* **5**, 2051-2060 (1985).
- Wobbe, C. R., Dean, F., Weissbach, L. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5710-5714 (1985).
- Waga, S., Bauer, G. & Stillman, B. *J. biol. Chem.* (in the press).
- Stillman, B. A. *Rev. Cell Biol.* **5**, 197-245 (1989).
- Challberg, M. D. & Kelly, T. J. A. *Rev. Biochem.* **58**, 671-717 (1989).
- Hurwitz, J., Dean, F. B., Kwong, A. D. & Lee, S.-H. *J. biol. Chem.* **265**, 18043-18046 (1990).
- Melendy, T. & Stillman, B. in *Nucleic Acids and Molecular Biology* Vol. 6 (eds Eckstein, F. & Lilly, D. M. J.) 129-158 (Springer, Berlin and Heidelberg, 1992).
- Borowiec, J. A., Dean, F. B., Bullock, P. A. & Hurwitz, J. *Cell* **60**, 181-184 (1990).
- Dean, F., Borowiec, J. A., Eki, T. & Hurwitz, J. *J. biol. Chem.* **267**, 14129-14137 (1992).
- Gannon, J. V. & Lane, D. P. *Nature* **329**, 456-458 (1987).
- Collins, K. L. & Kelly, T. J. *Molec. cell. Biol.* **11**, 2108-2115 (1991).
- Dornreiter, I. et al. *EMBO J.* **11**, 769-776 (1992).
- Melendy, T. & Stillman, B. *J. biol. Chem.* **268**, 3389-3395 (1993).
- Murakami, Y. & Hurwitz, J. *J. biol. Chem.* **268**, 11018-11027 (1993).
- Bullock, P. A., Seo, Y. S. & Hurwitz, J. *Molec. cell. Biol.* **11**, 2350-2361 (1991).
- Murakami, Y., Eki, T. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **89**, 952-956 (1992).
- Denis, D. & Bullock, P. A. *Molec. cell. Biol.* **13**, 2882-2890 (1993).
- Lee, S.-H., Kwong, A. D., Pan, Z.-Q. & Hurwitz, J. *J. biol. Chem.* **266**, 594-602 (1991).
- Tsurimoto, T. & Stillman, B. *J. biol. Chem.* **266**, 1950-1960 (1991).
- Tsurimoto, T. & Stillman, B. *J. biol. Chem.* **266**, 1961-1968 (1991).
- Tsurimoto, T. & Stillman, B. *EMBO J.* **8**, 3883-3889 (1989).
- Tsurimoto, T., Melendy, T. & Stillman, B. *Nature* **346**, 534-539 (1990).
- Weinberg, D. H. et al. *Proc. natn. Acad. Sci. U.S.A.* **87**, 8692-8696 (1990).
- Eki, T., Matsumoto, T., Murakami, Y. & Hurwitz, J. *J. biol. Chem.* **267**, 7284-7294 (1992).
- Goulian, M., Richards, S. H., Heard, C. J. & Bigsby, B. M. *J. biol. Chem.* **265**, 18461-18471 (1990).
- Ishimi, Y., Claude, A., Bullock, P. & Hurwitz, J. *J. biol. Chem.* **263**, 19723-19733 (1988).

- Turchi, J. J. & Bambara, R. A. *J. biol. Chem.* **268**, 15136-15141 (1993).
- Prelich, G. & Stillman, B. *Cell* **53**, 117-126 (1988).
- Tsurimoto, T. & Stillman, B. *Proc. natn. Acad. Sci. U.S.A.* **87**, 1023-1027 (1990).
- Eliasson, R. & Reichard, P. *J. biol. Chem.* **253**, 7469-7475 (1978).
- Nethanel, T. & Kaufmann, G. *J. Virol.* **64**, 5912-5918 (1990).
- Podust, V. N. & Hübscher, U. *Nucleic Acids Res.* **21**, 841-846 (1993).
- McHenry, C. S. *J. biol. Chem.* **266**, 19127-19130 (1991).
- Cha, T.-A. & Alberts, B. M. *Eukaryotic DNA Replication: Cancer Cells* **6**, 1-10 (Cold Spring Harbor Laboratory Press, New York, 1988).
- Kornberg, A. & Baker, T. *DNA Replication* (Freeman, New York, 1992).
- Marians, K. J. A. *Rev. Biochem.* **61**, 673-719 (1992).
- Young, M. C., Reddy, M. K. & von Hippel, P. H. *Biochemistry* **31**, 8675-8690 (1992).
- Konrad, E. B. & Lehman, I. R. *Proc. natn. Acad. Sci. U.S.A.* **71**, 2048-2051 (1974).
- Wang, T. S.-F. A. *Rev. Biochem.* **60**, 513-552 (1991).
- Roberts, J. D., Thomas, D. C. & Kunkel, T. A. *Proc. natn. Acad. Sci. U.S.A.* **88**, 3465-3469 (1991).
- Veaute, X. & Fuchs, R. P. P. *Science* **261**, 598-600 (1993).
- Araki, H. et al. *EMBO J.* **11**, 733-740 (1992).
- Burgers, P. M. J. *J. biol. Chem.* **266**, 22698-22706 (1991).
- Nishida, C., Reinhard, P. & Linn, S. *J. biol. Chem.* **263**, 501-510 (1988).
- Wang, Z., Wu, X. & Friedberg, E. C. *Molec. cell. Biol.* **13**, 1051-1058 (1993).
- Tsurimoto, T., Fairman, M. P. & Stillman, B. *Molec. cell. Biol.* **9**, 3839-3849 (1989).
- Fien, K. & Stillman, B. *Molec. cell. Biol.* **12**, 155-163 (1992).
- Tsurimoto, T. & Stillman, B. *Molec. cell. Biol.* **9**, 609-619 (1989).

ACKNOWLEDGEMENTS. We thank B. Alberts (University of California, San Francisco) for the gift of phage T4 DNA replication proteins; J. Turchi and R. Bambara for communication of unpublished results; L. Borzillo and N. Kessler for technical assistance; M. Ockler, P. Renna and J. Duffy for the photography and preparation of the figures; W. Herr and S. Bell for critical reading of this manuscript; and D. Costello for preparing it. This research was supported by a grant from the National Cancer Institute of the NIH.

Radar mapping of Mercury's polar anomalies

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GROUND-based radar observations of Mercury have revealed unusually strong, highly depolarized echoes from the north^{1,2} and south² poles. These anomalous echoes have been cited as evidence of polar ice deposits¹⁻⁵. Thermal studies³⁻⁵ suggest that the permanently shaded floors of large polar craters are cold enough to preserve water ice in a stable state over aeons, in spite of Mercury's proximity to the Sun. Here we present high-resolution radar maps of Mercury's polar regions, derived from delay-Doppler measurements. We have resolved the north and south polar anomalies into numerous crater-sized features, and we have been able to identify the source craters for many of these features after making small corrections to the pole positions on the Mariner-10 images. The coincidence with crater locations, together with other properties of the radar features, are consistent with the polar-ice hypothesis.

In a preliminary report of Arecibo radar results² we showed that the south polar anomaly was mostly confined to the large crater Chao Meng-Fu. At the north pole the evidence was not so tidy. The north polar anomaly was clearly too large to fit within a single crater and had an offset from the pole that placed most of it within the 'unphotographed' hemisphere (the night side at the time of the Mariner-10 encounters).

Since reporting these initial results we have vastly improved the quality of the polar radar images by making new observations and by reanalysing and summing the original data. The data were obtained with the Arecibo Observatory's S-band (2.4-GHz) radar in three observing campaigns during 1991-92. The observations used a phase-coded transmission with a 100- μ s delay resolution, giving a mapping resolution at the poles of 15 km in the line-of-sight (delay) coordinate. We achieved the same 15-km resolution in the transverse (Doppler) coordinate by quadrupling the length of the Fourier transform used in the spectral analysis from our original² 8,192 elements to 32,768 elements. (For details on the observations and analysis see ref. 2 and papers cited therein.) A mapping was done from delay-Doppler to planetary coordinates to give a polar projection image of radar cross-section per unit surface area (that is, specific cross-section σ_0) for each 25-min observing run. The polar images from various runs were then summed to reduce the statistical fluctuations from background noise. The north polar image (Fig. 1a) was formed by summing 27 observing runs; 16 of these runs were from the original eight observing dates² between 31 July and 29 August 1991, and the remaining 11 runs were from a new set of follow-up observations made on six dates between 19 July and 8 August 1992. The south polar image (Fig. 1b) is the sum of 16 runs obtained on eight dates between 14 March and 29 March 1992. Both images in Fig. 1 show the echo in the depolarized sense of circular polarization.

Figure 1a shows that most of the north polar anomaly is concentrated in crater-size (15-60 km) bright spots. We have plotted these features on the locating map in Fig. 2a and have given letter labels to those features that lie in the photographed hemisphere and to three prominent features in the unphotographed hemisphere. The brightest of the northern spots (D, E, H, J, K) roughly delineate the elongated and offset form of the unresolved anomaly as seen in the plane-of-sky images of Harmon and Slade². The image also shows a score of fainter spots as well as a diffuse patchiness that extends along the 330° W meridian. (Further observations may determine whether

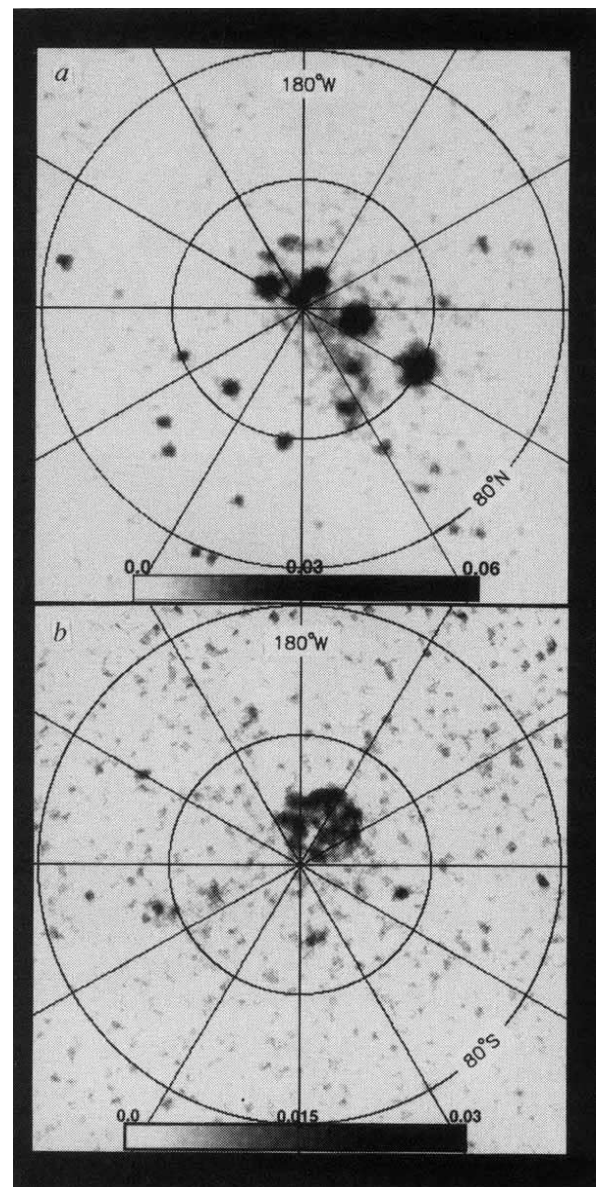


FIG. 1 Arecibo 2.4-GHz radar images of Mercury: a, north pole; b, south pole. The images show specific cross-section σ_0 (as given by the grey scale at the bottom of each panel) in the depolarized sense of circular polarization. The resolution is 15 km (0.35°). Note that the maximum value of σ_0 is 0.060 in a and 0.030 in b. The r.m.s. noise level is 3.5% of the maximum in a and 10.4% of the maximum in b.

the latter is truly diffuse or simply a dense collection of unresolved spots.) A comparison of the feature positions in Fig. 2a with the Mariner-10 image in Fig. 2b shows that by sliding the NASA/USGS coordinate grid in Fig. 2b down by 1.6° of latitude (along the 0° W meridian) one can get all of the features in the left half of the image to fall on or near known craters. The possibility that this is mere coincidence can be discounted, especially as many of these craters (for example, L-W) are located in an otherwise sparsely cratered region. The required 1.6° shift must represent an error in the NASA/USGS grid⁶⁻⁸ rather than in our radar grid because the delay-Doppler mapping gives positions relative to the true pole with an accuracy of $\sim 0.05^\circ$. Our results relocate the north pole to the southeastern rim of crater E (see Fig. 2b).

Figure 1b shows that the south polar echo is dominated by a 125-km-diameter circular feature (X) lying tangent to the pole. Sliding the NASA/USGS grid by 1.2° to the right (along the 90° W meridian) places feature X entirely inside Chao Meng-Fu

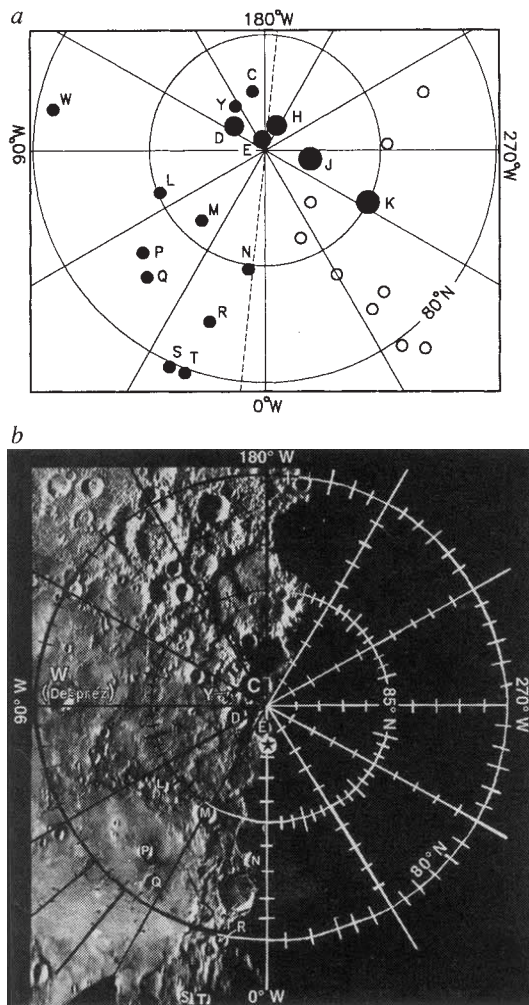


FIG. 2 Locating maps for north polar radar features. *a*, Locations of the letter-labelled features (filled circles) as well as some unlabelled features in the unphotographed hemisphere (open circles). The dashed line shows the location of the Mariner-10 terminator, the unphotographed hemisphere being to the right. *b*, Mariner-10 image of the north pole. Craters identified with radar features are given the same letter labels as in *a*. The coordinate grid in *b* is adapted from the NASA/USGS shaded relief maps⁶⁻⁸. There may be small discrepancies between the grid in *b* and the NASA/USGS grid due to distortions in the Mariner-10 image mosaics; there may therefore be differences between the crater positions in *b* and the crater coordinates listed in Table 1. Such distortions may also account for the tendency of the offsets between the radar features and source craters (for example, R, S, T) to increase near the bottom of the image. Our estimate of the true north pole position is shown in *b* (star).

crater and places features G, U and V inside other known craters (compare Fig. 3*a* and *b*). This grid shift also moves the south pole to a position which is in better agreement with the location of the Mariner-10 terminator (see Fig. 3*b*).

We stress that the proposed grid shifts represent readjustments of the NASA/USGS grid on the Mariner 10 images and not a new determination of the pole direction in the sky. The radar images (and grid) were calculated assuming zero polar obliquity and tests (in which the assumed pole direction was varied) showed that the zero-obliquity case gave minimum smearing of features in the summed images. Also, our proposed grid adjustments seem reasonable given that the NASA/USGS maps quote discrepancies of up to 40 km (1.0°) with respect to the Mercury control net and given that the control net itself has a standard error of 25 km (0.6°) at the north pole^{7,13}.

All of the geologically classified source craters in the north (C, D, E, M, N, R, T, W), as well as Chao Meng-Fu, have been

mapped^{8,9} as *c*₄-class (a USGS designation for craters that appear relatively pristine). Apparently the more degraded (and presumably older) craters do not contain near-surface deposits of radar-bright material.

In Table 1 we list the positions of the lettered radar features and their corresponding craters along with several parameters; (1) $\hat{\sigma}_p$, the peak feature reflectivity, (2) $\hat{\sigma}_a$, the feature's total depolarized cross-section divided by the mean area of crater floor that is both permanently shaded and radar-visible, (3) μ_c , the circular polarization ratio. Both reflectivity parameters ($\hat{\sigma}_p$, $\hat{\sigma}_a$) have been reduced to equivalent full-disk albedos to correct for incidence angle and enable comparison with other measurements. If the anomalies are enhanced back-scatter from ice then we might expect $\hat{\sigma}_p$ and $\hat{\sigma}_a$ to have values of a few tenths or so and $\mu_c > 1$ based on results from other icy bodies. (As the calculation of $\hat{\sigma}_a$ includes the stringent constraints imposed by the polar geometry, it serves as a particularly powerful test of the icy-crater hypothesis.)

The $\hat{\sigma}_p$ values indicate albedos of ~ 0.5 for the brighter features, which is two orders of magnitude higher than the disk-averaged depolarized albedo of the planet. For comparison, the depolarized albedos of the icy galilean satellites are in the range 0.4–1.6 (ref. 10) and the equivalent albedo of Mars' south polar icecap is 0.7 (refs 11, 12). The comparably high, but reasonable,

TABLE 1 Parameters of polar radar features/craters

Feature	Feature coordinates (°W, °N/°S)	Crater coordinates (°W, °N/°S)	$\hat{\sigma}_p$	$\hat{\sigma}_a$	μ_c
C	168W, 87.4N	133W, 89.1N	0.21	0.09	0.96 ± 0.14
D	128W, 88.3N	66W, 88.5N	0.60	0.61	1.25 ± 0.09
E	165W, 89.5N	12W, 88.8N	0.44	0.94	1.30 ± 0.10
G	75W, 86.0S	78W, 84.9S	0.30	0.14	1.34 ± 0.57
H	205W, 88.8N		0.53		1.15 ± 0.09
J	281W, 88.0N		0.48		1.38 ± 0.09
K	297W, 85.0N		0.27		1.30 ± 0.10
L	68W, 85.1N	52W, 84.2N	0.21	0.65	1.19 ± 0.25
M	42W, 85.9N	30W, 84.7N	0.28	0.28	1.24 ± 0.17
N	8W, 84.8N	4W, 83.2N	0.20	0.39	1.20 ± 0.18
P	50W, 83.1N	41W, 81.8N	0.15	0.34	1.50 ± 0.47
Q	43W, 82.5N	35W, 81.0N	0.12	0.29	1.74 ± 0.64
R	18W, 82.2N	12W, 80.0N	0.12	0.21	0.94 ± 0.22
S	24W, 79.8N	16W, 77.8N	0.09	0.27	1.62 ± 0.76
T	20W, 79.8N	13W, 78.1N	0.07	0.16	1.52 ± 0.73
U	13W, 87.1S	30W, 86.4S	0.21	0.07	1.25 ± 0.45
V	86W, 80.7S	86W, 79.8S	0.17	0.27	1.43 ± 1.16
W	101W, 80.7N	92W, 81.0N	0.28	0.45	1.27 ± 0.35
X	150W, 88.3S	133W, 87.5S	0.67	0.35	1.12 ± 0.09
Y	146W, 87.8N	112W, 88.4N	0.23	1.15	1.66 ± 0.45

The letter designations for C, D, E, G follow the crater naming convention in Paige *et al.*³; no radar features have been seen for their craters A, B, F. The only craters with proper IAU names are W (Despréz) and X (Chao Meng-Fu). The feature coordinates give the centre of the radar feature on the radar-based grid, while the crater coordinates give the position of the centre of the identified source crater on the NASA/USGS shaded relief maps⁶⁻⁹. Most of the difference between the two positions is apparently due to small pole-position errors or scale distortions in the shaded relief maps. The other parameters are; (1) $\hat{\sigma}_p$, the peak feature reflectivity, (2) $\hat{\sigma}_a$, the feature's total depolarized cross-section divided by the mean area of crater floor that is both permanently shaded and radar-visible, and (3) μ_c , the feature's total depolarized cross-section divided by its total polarized cross-section. Both $\hat{\sigma}_p$ and $\hat{\sigma}_a$ are given as equivalent full-disk albedos; they were calculated from the specific cross section σ_0 using $\hat{\sigma} = 2\sigma_0/(n+1) \cos^2 \theta$ and assuming an $n = 3/2$ cosine scattering law (θ is incidence angle). The $\hat{\sigma}_a$ calculation assumed that the craters have flat floors, perpendicular walls, and a depth/diameter relation $d = 0.410D^{0.490}$ ($D < 30$ km) and $d = 0.353D^{0.496}$ ($D > 30$ km). Also, the shadow calculation assumed the Sun to be a point source, so some penumbral illumination is included. $\hat{\sigma}_p$ is 5–29 standard deviations above the noise background for the northern features and 5–10 standard deviations above the noise for the southern features. The error quoted for μ_c is one standard deviation.

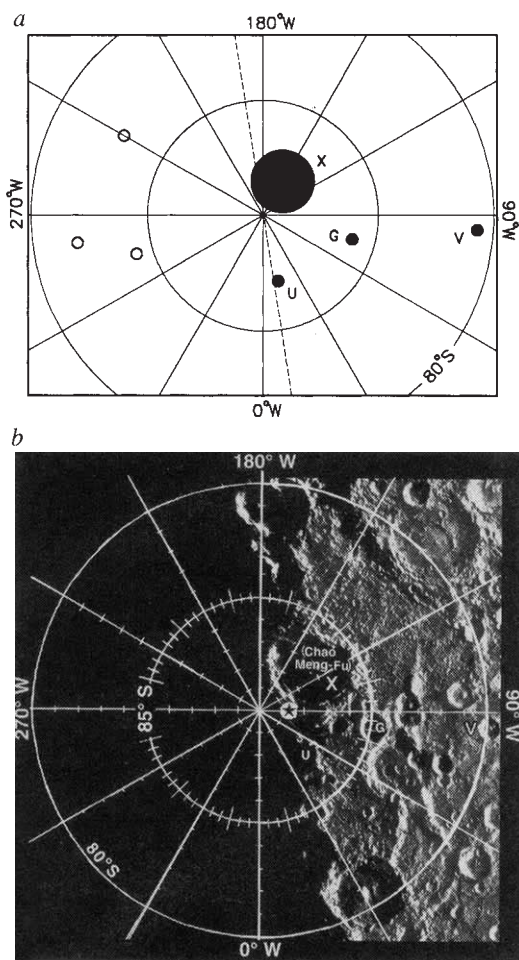


FIG. 3 Locating maps for south polar radar features. *a*, Locations of the letter-labelled features (filled circles) as well as some unlabelled features in the unphotographed hemisphere (open circles). The dashed line shows the location of the Mariner-10 terminator, the unphotographed hemisphere being to the left. *b*, Mariner-10 image of the south pole. There may be small discrepancies between the grid in *b* and the NASA/USGS grid due to distortions in the Mariner-10 image mosaics; there may therefore be differences between the crater positions in *b* and the crater coordinates listed in Table 1. Our estimate of the true south pole position is shown in *b* (star).

values of $\hat{\sigma}_a$ suggest that all of the identified source craters have enough shaded, radar-visible area to account for the high cross-sections. This seems to be the case even for features with latitudes as low as 80–81° (features S, T, V, W). However, permanent shadowing alone may not guarantee temperatures low enough to sustain ice^{3,4}, and additional theoretical work should be done to evaluate the plausibility of ice existing at these lower latitudes. Finally, Table 1 shows that the radar polarization of the features tends to be 'inverted' ($\mu_c > 1$); for the strongest of the features the measured inversion is statistically significant. Such polarization inversion is consistent with the X-band radar results for Mercury's north polar anomaly¹ and is reminiscent of the anomalous radar polarization of the icy galilean satellites¹⁰ and Mars' southern icecap¹¹.

Our new images prove the connection between the radar anomalies and polar craters, and provide strong support for the notion that the radar-bright material is concentrated in permanently shaded crater floors. The most plausible model still seems to be one in which shaded crater floors act as cold traps for water ice, thick deposits of which provide a low-loss medium for enhanced volume backscatter of radio waves. Conclusive proof of the existence of polar ice will require spacecraft missions equipped with ultraviolet or neutron spectrometers and, poss-

ibly, sampling penetrators. The next mission to Mercury could, at the very least, provide the photomapping necessary to (1) corroborate the cartographic grid refinements proposed here, and (2) identify source craters for those radar-bright spots which are presently located in unphotographed terrain. □

Received 22 February; accepted 5 April 1994.

1. Slade, M. A., Butler, B. J. & Muhleman, D. O. *Science* **258**, 635–640 (1992).
2. Harmon, J. K. & Slade, M. A. *Science* **258**, 640–643 (1992).
3. Paige, D. A., Wood, S. E. & Vasavada, A. R. *Science* **258**, 643–646 (1992).
4. Ingersoll, A. P., Svitek, T. & Murray, B. C. *Icarus* **100**, 40–47 (1992).
5. Butler, B. J., Muhleman, D. O. & Slade, M. A. *J. geophys. Res.* **98**, 15003–15023 (1993).
6. Davies, M. E., Dwornik, S. E., Gault, D. E. & Strom, R. G. *Atlas of Mercury* (NASA, Washington DC, 1978).
7. *Shaded Relief Map of Mercury* (USGS Map I-1149, US Geological Surv., Reston, Virginia, 1979).
8. Grollier, M. J. & Boyce, J. M. *Geologic Map of the Borealis Region of Mercury* (USGS Map I-1660, US Geological Surv., Reston, Virginia, 1984).
9. Strom, R. G., Malin, M. C. & Leake, M. A. *Geologic Map of the Bach Region of Mercury* (USGS Map I-2015, US Geological Surv., Reston, Virginia, 1990).
10. Ostro, S. J. et al. *J. geophys. Res.* **97**, 18227–18244 (1992).
11. Muhleman, D. O., Butler, B. J., Grossman, A. W. & Slade, M. A. *Science* **253**, 1508–1513 (1991).
12. Harmon, J. K., Slade, M. A. & Hudson, R. S. *Icarus* **98**, 240–253 (1992).
13. Davies, M. E. & Batson, R. M. *J. geophys. Res.* **80**, 2417–2430 (1975).

ACKNOWLEDGEMENTS. We thank R. Thomas, A. Venkataraman and P. Perillat for their assistance with computing. Ephemerides for the radar observations were supplied by the Center for Astrophysics. M.J.D. and J.M.J. were supported by the Arecibo summer student programme under a grant from the REU program of the US NSF. Part of this work was carried out under contract with NASA. The National Astronomy and Ionosphere Center (Arecibo Observatory) is operated by Cornell University under a cooperative agreement with the US NSF and with support from NASA.

Experimental observation of self-replicating spots in a reaction–diffusion system

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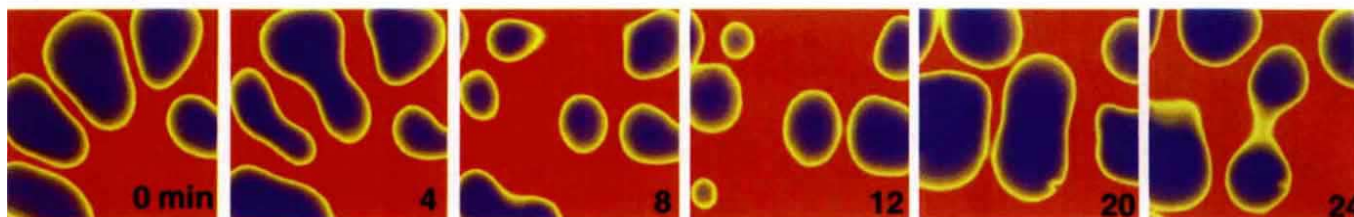
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IN his classic 1952 paper, Turing¹ suggested a possible connection between patterns in biological systems and patterns that could form spontaneously in chemical reaction–diffusion systems. Turing's analysis stimulated considerable theoretical research on mathematical models of pattern formation, but Turing-type patterns were not observed in controlled laboratory experiments until 1990^{2,3}. Subsequently there has been a renewed interest in chemical pattern formation and in the relationship of chemical patterns to the remarkably similar patterns observed in diverse physical and biological systems⁴. Numerical simulations of a simple model chemical system have recently revealed spot patterns that undergo a continuous process of 'birth' through replication and 'death' through overcrowding⁵. Here we report the observation of a similar phenomenon in laboratory experiments on the ferrocyanide–iodate–sulphite reaction. Repeated growth and replication can be observed for a wide range of experimental parameters, and can be reproduced by a simple two-species model, suggesting that replicating spots may occur in many reaction–diffusion systems.

The laboratory chemical patterns form in a thin transparent gel (0.4 mm thick, 22 mm diameter) whose bottom surface is in contact with a well-stirred reservoir (3.0 ml volume) that is continuously refreshed with the reagents of the reaction⁶. Transitions are studied by changing the input ferrocyanide concentration with other parameters held fixed. In the parameter range studied, the concentrations in the reservoir are independent of time. Spatial variations of the chemical concentrations in the plane of the gel—that is, chemical patterns—are viewed with a video camera in reflected light in a band centred at 420 nm, where ferrocyanide absorbs strongly.

Laboratory experiment



Numerical simulation

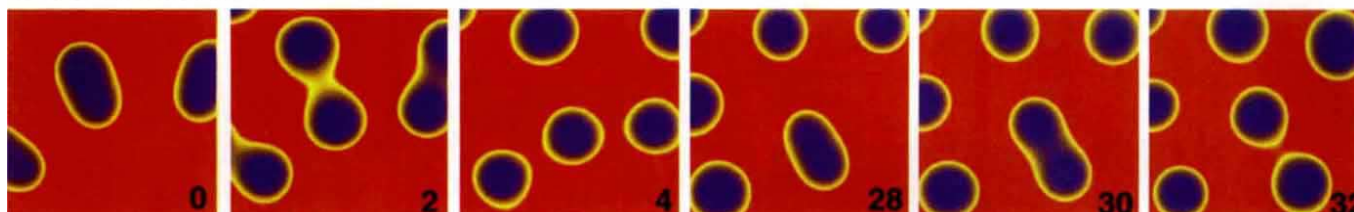


FIG. 1 The upper row of images shows the evolution of a chemical pattern observed in a laboratory experiment, and the lower row shows similar behaviour found in a numerical simulation of a reaction-diffusion model (see text). The behaviour illustrated here continues indefinitely, as long as the reactor conditions are maintained. In the experiment, blue (red) represents a state of high (low) pH, and the domain size is 7 mm $\times$ 7 mm. The concentrations of the reagents fed into the reservoir that is in contact with the gel reactor are (in mM):

[NaO₃] = 75.0, [Na₂SO₃] = 89.0, [H₂SO₄] = 3.6, [NaOH] = 0.25 and [K₄Fe(CN)₆ · 3H₂O] = 36.4. The total input flow rate is 86.4 ml h⁻¹, and the reactor temperature is 30 °C. The dimensionless parameter values for the numerical simulation are $A=0.02$, $B=0.079$ and $D_U=2 \times D_V=2.0 \times 10^{-5}$; each panel shows a 150 $\times$ 150 pixel region from a domain of 512 $\times$ 512 pixels. The images from the simulation are coded red ($U=1$, $V=0$) and blue ($U=0.3$, $V=0.25$) and are labelled with dimensionless time/100.

Numerical simulations were conducted for a two-dimensional reaction-diffusion model system with the reaction $U + 2V \rightarrow 3V$ coupled to a reservoir held at the fixed concentrations (U_0 , V_0) (ref. 5):

$$\frac{\partial U}{\partial t} = D_U \nabla^2 U - UV^2 + A(U_0 - U)$$

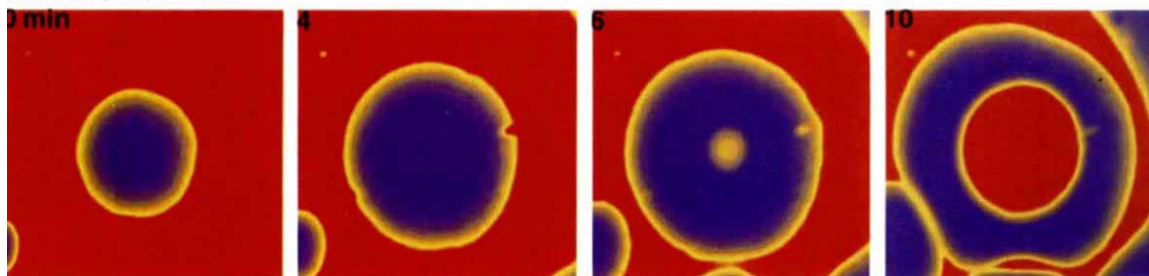
$$\frac{\partial V}{\partial t} = D_V \nabla^2 V + UV^2 + B(V_0 - V)$$

where ($U_0=1$, $V_0=0$). The parameters A and B represent the strengths of the coupling of U and V to the reservoir, D_U and D_V are the diffusion coefficients, and t is time. In a homogeneous

system our model exhibits oscillations and bistability, just as found in experiments and models for the well-studied Belousov-Zhabotinsky, chlorite-iodide-malonic acid, and ferrocyanide-iodate-sulphite⁷ reactions. The differences in the pattern-forming behaviour of these systems stem in part from differences in the relative diffusion coefficients of different species in the reactions.

Numerical simulations of this reaction-diffusion model were performed in an attempt to find stationary lamellar patterns like those observed in an earlier experiment on the ferrocyanide-iodate-sulphite reactions⁶. The model yielded not only lamellar patterns but also self-replicating spots, as shown in Fig. 1, and other patterns⁵. A subsequent search for spots in the experiments

Laboratory experiment



Numerical simulation

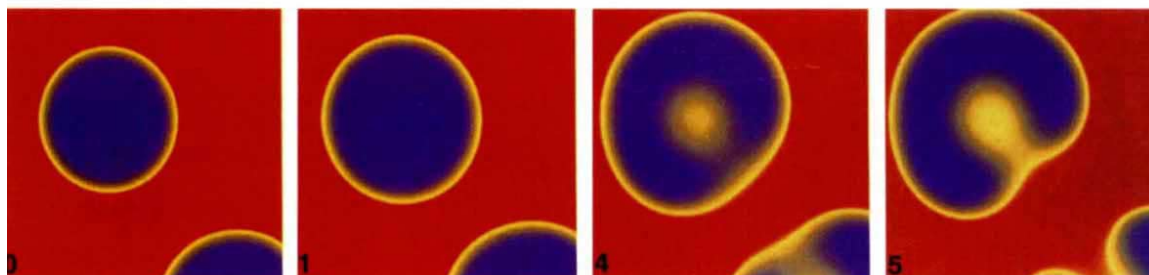


FIG. 2 Sequences showing the transition from a spot to an annulus in the experiments (upper row, 10 mm $\times$ 10 mm domain) and simulations

(lower row, 120 $\times$ 120 pixel domain). The parameter values are the same as in Fig. 1.

FIG. 3 Evolution of a square perturbation ($U=0.5$, $V=0.25$) of the initially homogeneous state ($U=1$, $V=0$). The initial square (a) is 50×50 pixels in a reaction domain of 512×512 pixels. a, b, c and d, Images corresponding to $t=0$, 4.2, 7.0 and 9.8 (dimensionless time/100), respectively (black, $U < 0.44$).

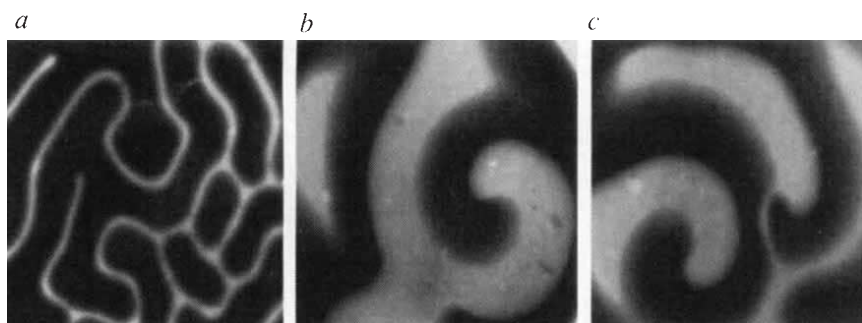
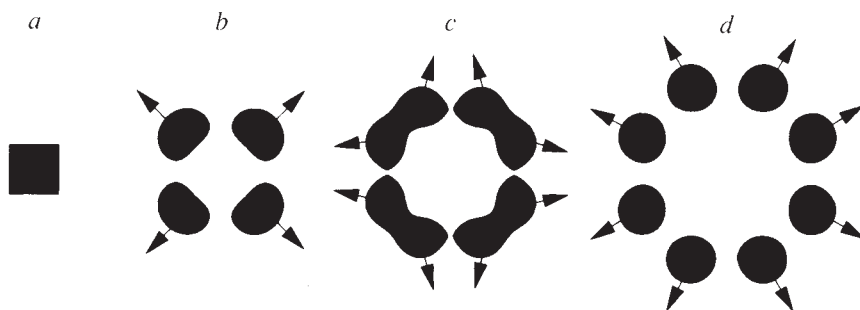


FIG. 4 Patterns formed at lower ferrocyanide concentrations than in Figs 1 and 2. a, Lamellar pattern; b, rotating spiral waves with wavefront annihilation; c, rotating spiral waves with wavefront repulsion. The high- and low-pH regions show as dark and light, respectively. The ferrocyanide concentrations in the feed are 14.2, 25.0 and 30.0 mM, respectively. Each frame is $18 \text{ mm} \times 18 \text{ mm}$.

produced not only spots but also annular patterns, as shown in Fig. 2. The annular patterns were then found in the model as well.

Spots do not form spontaneously from a uniform state in either the experiments or simulations. But once they have been initiated by a perturbation, the replicating spot patterns are self-sustaining. In the experiments, spots can be initiated by a perturbation using ultraviolet light or an inhomogeneity at the boundary.

Insight into the self-replicating behaviour can be gained from consideration of the evolution of spots in the model⁶. A spot, a region of high V and low U , is maintained by the diffusive flux of U (the more rapidly diffusing species) into the spot. Inside the spot, U is converted to V , while the spot loses V through diffusion into the reservoir and the spot's surroundings. Whether a spot grows or shrinks depends on the magnitude of the flux F of U through the spot's boundary. There is a critical flux of U , F_c , above (below) which the boundary moves toward the region of higher (lower) U .

The spot replication process is illustrated in Fig. 3, which shows the evolution of a square-shaped perturbation of the homogeneous state. The initial growth is largest in the corners of the square where the curvature is largest. The flux of U into the centre of the square is insufficient to maintain this region in the high- V state, and it collapses, leaving elongated spots at the corners of the square (Fig. 3b). These spots move outward along the diagonals because $F > F_c$ from the region outside the square while $F < F_c$ from inside. The decrease of U in the centre of a spot as it grows leads to a rapid decrease in V in the spot's centre. The spot then grows more rapidly in the direction orthogonal to the diagonals than along the diagonals. Hence the spot starts to pinch (Fig. 3c) and eventually divides into two daughter spots (Fig. 3d). This replication process happens repeatedly.

Spots repel each other when they approach very close because the region between them becomes depleted in U . If spots become overcrowded, many of them die. The flux of U into a surviving isolated spot is almost isotropic, and the spot expands until the flux of U is insufficient to maintain the spot centre in the high- V state. Then the centre collapses, leaving an annular ring, as shown in Fig. 2. These annular structures eventually break into pieces through interactions with neighbours.

We now describe the patterns observed for ferrocyanide concentrations lower and higher than the range in which spots

form. At low ferrocyanide concentrations, domains of low pH spontaneously emerge from a uniform high-pH state. Each domain grows, and then shrinks to a thin stripe with a well-defined thickness. These stripes move and interact until the entire domain is filled with a stationary lamellar pattern, as shown in Fig. 4a. Although the pattern is irregular, there are well-defined length scales associated with the high- and low-pH regions. The lamellar pattern looks similar to those previously observed⁶, but the previous patterns required a finite-amplitude perturbation for their initiation.

When the ferrocyanide concentration is increased, spiral waves form (Fig. 4b). As in the classic Belousov-Zhabotinsky reactions^{9,10}, these spiral waves annihilate upon collision, forming cusps (see the upper-right corner in Fig. 4b). The characteristic size of these spirals is much larger than the replicating spots.

When the ferrocyanide concentration is further increased in the direction that yields the replicating spots, the behaviour of two colliding fronts becomes quite different from that in excitable media: instead of annihilating on collision, fronts approach one another but remain separated by a sustained narrow zone of low pH; see Fig. 4c. Then one (or both) of the waves breaks and the broken ends become spiral tips, a process that leads to a large number of spiral tips. At yet higher ferrocyanide concentration, the new front interactions dominate, leading to many small wave segments, and eventually the system enters the replicating spot state illustrated in Fig. 1. For ferrocyanide concentration values beyond those of the replicating spot regime, the system goes into the homogeneous low-pH state. The transitions from Fig. 4b to c and from Fig. 4c to Fig. 1 are not sharp. In parameter ranges near each transition, both types of patterns can occur in different parts of the gel reactor.

The annihilation of colliding fronts is also found in the model, but for lower values of B than used in the simulations we have described. Smaller values of B sustain higher values of U between two approaching fronts, enough to maintain the propagation of fronts until they collide and annihilate.

In conclusion, spot replication and annuli are observed for a wide parameter range in the experiments on the ferrocyanide-iodate-sulphite reaction, and these observations are qualitatively described by a simple two-species model, as Figs 1 and 2 illustrate. The growth and interaction of spots and annuli lead to very complicated spatiotemporal patterns that continue to evolve indefinitely. Replicating spots, along with the previously

1. Turing, A. M. *Phil. Trans. R. Soc. B* **327**, 37–72 (1952).
2. Castets, V., Dulos, E., Boissonade, J. & De Kepper, P. *Phys. Rev. Lett.* **64**, 2953–2956 (1990).
3. Ouyang, Q. & Swinney, H. L. *Nature* **352**, 610–612 (1991).
4. Cross, M. C. & Hohenberg, P. C. *Rev. mod. Phys.* **65**, 851–1112 (1993).
5. Pearson, J. E. *Science* **261**, 189–192 (1993).
6. Lee, K. J., McCormick, W. D., Ouyang, Q. & Swinney, H. L. *Science* **261**, 192–194 (1993).
7. Gáspár, V. & Showalter, K. J. *phys. Chem.* **94**, 4973–4979 (1990).
8. Reynolds, W. N., Pearson, J. E. & Ponce-Dawson, S. *Phys. Rev. Lett.* **72**, 2797–2800 (1994).
9. Zaikin, A. N. & Zhabotinskii, A. M. *Nature* **225**, 535–537 (1970).
10. Winfree, A. T. *Science* **175**, 634–636 (1972).
11. De Kepper, P., Perraud, J. J., Rudovics, B. & Dulos, E. *Int. J. Bifurcation Chaos* (submitted).
12. Petrich, D. M. & Goldstein, R. E. *Phys. Rev. Lett.* **72**, 1120–1123 (1994).
13. Hagberg, A. & Meron, E. *Phys. Rev. Lett.* **72**, 2494–2497 (1994).

Chemical self-replication of palindromic duplex DNA

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Figure 1a depicts the self-replication cycle that we have designed, which is based on the well-known principles of triple-strand^{10,11} (Watson–Crick–Hoogsteen) and double-strand (Watson–Crick) DNA formation. It was anticipated that the relatively weaker Hoogsteen binding involved in triple helices would allow release of the newly generated strand from a double helical

a

The diagram shows the following steps:

- Step 1:** Duplex DNA is converted to a single-strand DNA fragment (lacking P-O bond(s)) via [ligation].
- Step 2:** The single-strand DNA fragment is assembled into a triplex DNA structure (lacking P-O bond(s)).
- Step 3:** The triplex DNA is dissociated into a duplex DNA (single-strand DNA template) and a single-strand DNA fragment.
- Step 4:** The single-strand DNA fragment is assembled into a duplex DNA (lacking P-O bond(s)).
- Step 5:** The duplex DNA (lacking P-O bond(s)) is converted back to a duplex DNA via [ligation].
- Step 6:** The duplex DNA is converted back to a duplex DNA via [ligation].

b

Chemical structures illustrating the formation of a triplex DNA structure from duplex DNA:

Initial duplex DNA structure (S₁, S₂):

$$\begin{array}{l} 5' \text{ P-C-C-C-T-T-C-C-T-C-T-T-T-T-T-C-T-C-C-T-T-C-C-C-OH } 3' \\ 3' \text{ HO-G-G-G-A-A-G-G-A-G-A-A-A-A-A-G-A-G-G-A-A-G-G-G-P } 5' \end{array} \quad \{S_1, S_2\}$$

Intermediate structure (S₁, S₂, F₁, F₂):

$$\begin{array}{l} 5' \text{ P-C-C-C-T-T-C-C-T-C-T-T-T-T-T-C-T-C-C-T-T-C-C-C-OH } 3' \\ 3' \text{ HO-G-G-G-A-A-G-G-A-G-A-A-A-A-A-G-A-G-G-A-A-G-G-G-P } 5' \\ 3' \text{ HO-C-C-C-T-T-C-T-C-T-T-T-T-T-T-C-T-C-C-T-T-C-C-C-P } 5' \end{array} \quad \{S_1, S_2, F_1, F_2\}$$

Structure with a phosphate group (S₁, S₂, S₁^{*}):

$$\begin{array}{l} 5' \text{ P-C-C-C-T-T-C-C-T-C-T-T-T-T-T-C-T-C-C-T-T-C-C-C-OH } 3' \\ 3' \text{ HO-G-G-G-A-A-G-G-A-G-A-A-A-A-A-G-A-G-G-A-A-G-G-G-P } 5' \\ 3' \text{ HO-C-C-C-T-T-C-T-C-T-T-T-T-T-T-C-T-C-C-T-T-C-C-C-P } 5' \end{array} \quad \{S_1, S_2, S_1^*\}$$

Structure with a phosphate group (S₁, S₂):

$$\begin{array}{l} 5' \text{ P-C-C-C-T-T-C-C-T-C-T-T-T-T-T-C-T-C-C-T-T-C-C-C-OH } 3' \\ 3' \text{ HO-G-G-G-A-A-G-G-A-G-A-A-A-A-A-G-A-G-G-A-A-G-G-G-P } 5' \end{array} \quad \{S_1, S_2\}$$

Structure with a phosphate group (S₁^{*}, F₃, F₄):

$$\begin{array}{l} 5' \text{ P-C-C-C-T-T-C-C-T-C-T-T-T-T-T-C-T-C-C-T-T-C-C-C-OH } 3' \\ 3' \text{ HO-G-G-G-A-A-G-G-A-G-A-A-A-A-A-G-A-G-G-A-A-G-G-G-P } 5' \\ 3' \text{ HO-C-C-C-T-T-C-T-C-T-T-T-T-T-T-C-T-C-C-T-T-C-C-C-P } 5' \end{array} \quad \{S_1^*, F_3, F_4\}$$

Structure with a phosphate group (S₁, S₂):

$$\begin{array}{l} 5' \text{ P-C-C-C-T-T-C-C-T-C-T-T-T-T-T-C-T-C-C-T-T-C-C-C-OH } 3' \\ 3' \text{ HO-G-G-G-A-A-G-G-A-G-A-A-A-A-A-G-A-G-G-A-A-G-G-G-P } 5' \end{array} \quad \{S_1, S_2\}$$

Structure with a phosphate group (S₁^{*}, S₂^{*}):

$$\begin{array}{l} 5' \text{ P-C-C-C-T-T-C-C-T-C-T-T-T-T-T-C-T-C-C-T-T-C-C-C-OH } 3' \\ 3' \text{ HO-G-G-G-A-A-G-G-A-G-A-A-A-A-A-G-A-G-G-A-A-G-G-G-P } 5' \\ 3' \text{ HO-C-C-C-T-T-C-T-C-T-T-T-T-T-T-C-T-C-C-T-T-C-C-C-P } 5' \end{array} \quad \{S_1^*, S_2^*\}$$

template¹², thus ensuring, in combination with double-strand formation, continuous production of new copies. According to this scheme, duplex DNA serves, at an appropriately acidic pH, as a template to attract complementary single-strand DNA fragments, pre-organizing them along its major groove and orienting them in the right direction for ligation (1→2, Fig. 1a)¹³. A suitable chemical reagent then brings about P-O bond formation¹³ thus forming a new strand, identical with one of the two strands of the original duplex (2→3). Raising the pH (pH driven) or adding an excess of oligonucleotides complementary to the newly formed strand drives the equilibrium away from the triplex structure, releasing the single strand (3→4), thus allowing it to serve as a template for duplex formation in the presence of complementary single-strand DNA fragments (4→5)¹⁴⁻¹⁸. Finally, a chemical reagent causing P-O bond formation¹⁸ completes the cycle by generating a new strand identical to the second strand of the original DNA in an overall process that accomplishes replication of the original duplex (5→1, 6). This scheme, of course, requires symmetrical sequences, otherwise the product of the cycle is of the retro-sense. In the latter case (non-palindromic sequences) a second cycle and appropriate substrate fragments

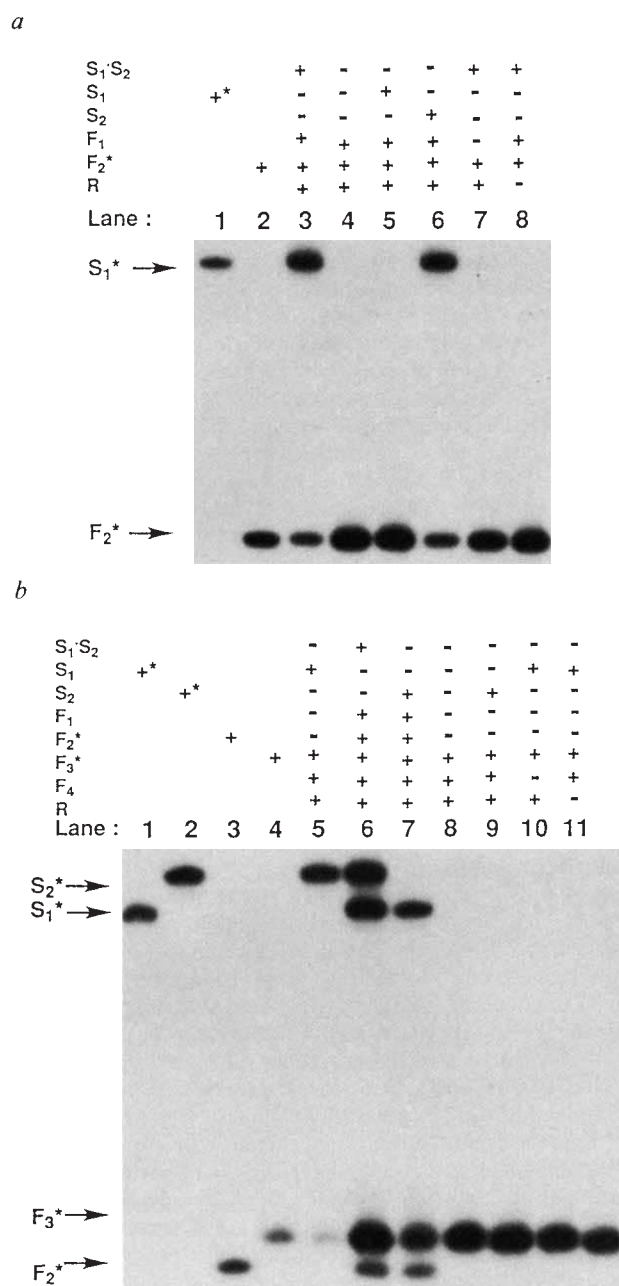


FIG. 2 Replication of duplex oligodeoxyribonucleotide ($S_1 \cdot S_2$) by varying pH (6.0 $\rightarrow$ 7.0). Autoradiograms of high-resolution, denaturing 20% polyacrylamide gels. **a**, First half of the cycle; **b**, second half of the cycle. **METHODS.** Radioactive fragments were prepared by phosphorylation of synthetic oligonucleotides at their 5' end with polynucleotide kinase and [γ - 32 P]ATP; asterisk indicates radioactivity incorporated to the extent unlabelled: labelled $\approx$ 100. R is the reagent *N*-cyanoimidazole. Reactions were processed after termination by cooling in dry ice, evaporation to dryness, dissolving in loading buffer and denaturation. **a**, First half of the cycle. Demonstration of template ($S_1 \cdot S_2$)-directed formation of copies of S_1 strand from oligodeoxyribonucleotide fragments F_1 and F_2 . Lane 1, S_1^* ; lane 2, F_2^* . Lanes 3–8 represent reaction mixtures, each of which was obtained by incubation of 100 mM imidazole-HCl (initial pH 6.0), 10 μ M $ZnCl_2$, 100 mM NaCl, 40 mM *N*-cyanoimidazole and oligodeoxyribonucleotides in a total volume of 30 μ l at 20 $^\circ$ C for 72 h. Lanes: 3, 10 μ M annealed $S_1 \cdot S_2$, 10 μ M F_1 and 10 μ M F_2^* ; 4, 10 μ M F_1 and 10 μ M F_2^* ; 5, 10 μ M S_1 , 10 μ M F_1 and 10 μ M F_2^* ; 6, 10 μ M S_2 , 10 μ M F_1 and 10 μ M F_2^* ; 7, 10 μ M $S_1 \cdot S_2$ and 10 μ M F_2^* ; 8, as lane 3, except for the absence of ligating agent *N*-cyanoimidazole. **b**, Second half of the cycle. Newly formed copies of S_1 released from template by increasing the pH to 7.0, directed formation of S_2 strand from oligodeoxyribonucleotides F_3 and F_4 . Lane 1, S_1^* ; lane 2, S_2^* ; lane 3, F_2^* ; lane 4, F_3^* . Lanes 5 and 8–11 represent reaction mixtures obtained by incubation of 100 mM imidazole (initial pH 7.0), 10 μ M $ZnCl_2$, 100 mM NaCl, 80 mM *N*-cyanoimidazole and oligodeoxyribonucleotides in a total volume of 30 μ l at 20 $^\circ$ C for 72 h. Lanes: 5, 5 μ M S_1 , 5 μ M F_3^* and 5 μ M F_4 ; 8, 5 μ M F_3^* and 5 μ M F_4 ; 9, 5 μ M S_2 , 5 μ M F_3^* and 5 μ M F_4 ; 10, 5 μ M S_1 and 5 μ M F_3^* ; 11, as in lane 5, except for the absence of ligating agent *N*-cyanoimidazole. The reaction mixtures of lanes 6 and 7 were prepared from 15 μ l of the original solutions represented by lanes 3 and 6 in **a** (after 72 h incubation) and appropriate addition of reagents, bringing the total volume to 30 μ l containing 100 mM imidazole-HCl (initial pH 7.0), 10 μ M $ZnCl_2$, 100 mM NaCl, 80 mM *N*-cyanoimidazole and oligonucleotides. After incubation for 72 h at 20 $^\circ$ C, reactions were terminated by cooling in dry ice, samples were lyophilized, dissolved in loading buffer and denatured. Lane 6, 5 μ M F_3^* , 5 μ M F_4 and 15 μ l of solution, corresponding to lane 3 in **a**; lane 7, 5 μ M F_3^* , 5 μ M F_4 and 15 μ l of solution, corresponding to lane 6 in **a**.

will be required to produce an identical copy of the original template. It should also be noted that the present triplex replication scheme requires at least one of the strands to be an all-purine sequence.

Figure 1b summarizes the experiments leading to replication of a palindromic 24-monomer duplex DNA fragment ($S_1 \cdot S_2$). Fragments F_1 and F_2^* (chosen to be complementary to S_2) were added to the DNA duplex (pre-annealed in the presence of 100 mM NaCl by incubation at 90 $^\circ$ C for 3 min followed by slow cooling to 20 $^\circ$ C over 1 h) in a pH 6.0 buffer solution (imidazole/HCl) to form the triplex ($S_1 \cdot S_2 \cdot F_1, F_2^*$) in which one P–O bond is missing in strand F_1, F_2^* . *N*-cyanoimidazole¹⁸ (cyanogen bromide¹⁸ also works, but less efficiently) was then added to the mixture, ligating the two fragments of the third strand by P–O bond formation and leading to the complete triple helix ($S_1 \cdot S_2 \cdot S_1^*$).

The products were analysed by denaturing gel electrophoresis. As shown in Fig. 2a (lane 3), a new strand, $F_1-F_2^*$, which is identical to S_1^* , was formed with high yield (80%). Appropriate control experiments revealed no S_1 strand formation in the

absence of ($S_1 \cdot S_2$) template (lane 4, Fig. 2a), or in the presence of S_1 (lane 5, Fig. 2a), but efficient S_1^* strand formation in the presence of S_2 template as expected (lane 6, Fig. 2a). No condensation was apparent in the absence of F_1 (lane 7, Fig. 2a). Furthermore, the requirement for *N*-cyanoimidazole for the formation of S_1 was confirmed (lane 8, Fig. 2a). The second strand (S_2) of the duplex was replicated in the same reaction mixture at pH 7.0 using the newly generated first strand ($F_1-F_2^*$) ($\equiv S_1^*$) as a template. Thus, fragments F_3^* and F_4 (complementary to F_1 and F_2^* respectively) were added to form the 'disconnected' duplex ($S_1^* \cdot F_3^*, F_4$), which is lacking a P–O bond between F_3^* and F_4 . Addition of *N*-cyanoimidazole (or cyanogen bromide) brought about smooth coupling of the two fragments as expected. Because these sequences are palindromic, the newly formed DNA duplex copy ($F_1-F_2^* \cdot F_3^*-F_4$) ($\equiv S_1^* \cdot S_2^*$) (lane 6, Fig. 2b; 50% yield based on the newly formed template, 40% yield based on the original duplex) is identical with the original duplex ($S_1 \cdot S_2$). The results of appropriate control experiments are shown in Fig. 2b and are in accord with the proposed reactions. Digestion of $F_1-F_2^*$ ($\equiv S_1^*$) and $F_3^*-F_4$ ($\equiv S_2^*$) with phosphodiesterase I revealed the presence of a 3', 5'-phosphodiester bond, thus confirming the expected orientation of fragments F_1, F_2, F_3 and F_4 on the templates. It was of interest to note in these experiments several slower moving bands (totalling $\sim$ 5% of radioactivity and reaching $\sim$ 30% with increasing amount of *N*-cyanoimidazole, not shown in gel). These products, assumed to be 36- and higher polymers, are presumed to be formed by ligation at the ends of the triple helices, a phenomenon that may be of special significance in considering elongation of DNA.

Self-replication of a palindromic duplex DNA ($S_1 \cdot S_2$) was also demonstrated at the constant pH of 6.15. At this pH, it was anticipated that the equilibrium¹² between the triplex structure

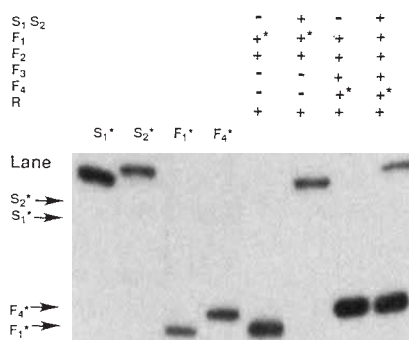


FIG. 3 Replication of duplex oligodeoxyribonucleotide ($S_1 \cdot S_2$) at constant pH 6.15. Autoradiogram of high-resolution, denaturing 20% polyacrylamide gel.

METHODS. Radiolabelling and reaction processing were carried out as for Fig. 3. R is the reagent *N*-cyanoimidazole. Strand sequences were the same as in Fig. 2 apart from the absence of phosphate at the 5' end of S_1 , S_2 , F_2 and F_3 . Lane 1, S_1^* ; lane 2, S_2^* ; lane 3, F_1^* ; lane 4, F_2^* ; lane 5, 100 mM imidazole-HCl, 5 μ M $ZnCl_2$, 100 mM NaCl, 20 mM *N*-cyanoimidazole, 20 μ M annealed ($S_1 \cdot S_2$) (2 nmol), 24 μ M F_1^* (2.4 nmol) and 24 μ M F_2 (2.4 nmol) in a total volume of 100 μ l, incubated at 20 °C for 72 h; lane 6, same as in lane 5 apart from the absence of ($S_1 \cdot S_2$). Lane 7, as lane 6 except that only unlabelled F_1 was used in the first half of the cycle; F_3 (12 nmol), F_4^* (12 nmol), buffer and water were added and the mixture was incubated for 10 h at 20 °C before *N*-cyanoimidazole was added, giving a total volume of 200 μ l (pH 6.15) and containing 100 mM imidazole-HCl, 5 μ M $ZnCl_2$, 100 mM NaCl, 20 mM *N*-cyanoimidazole and oligonucleotides. The reaction mixture was incubated at 20 °C for a further 72 h. Lane 8, as lane 7 except for the initial absence of ($S_1 \cdot S_2$) and the use of only 2.4 μ M (0.48 nmol) of each F_1^* and F_2 . Yield of generated duplex DNA was determined by counting radioactivity of the bands corresponding to S_1^* and F_1^* (lane 6) and S_2^* and F_4^* (lane 8).

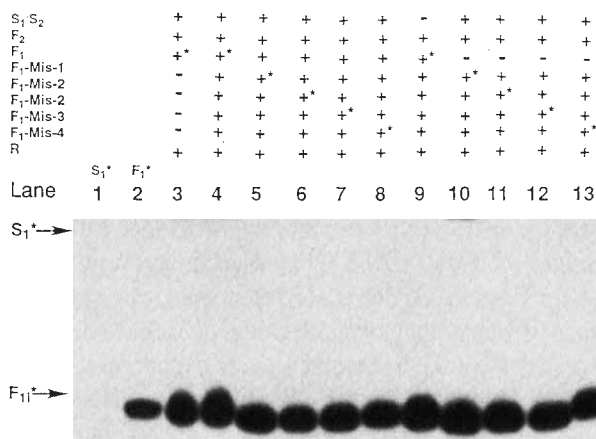


FIG. 4 Selection and mutation in self-replication of duplex oligodeoxyribonucleotide ($S_1 \cdot S_2$). Autoradiogram of high resolution, 20% polyacrylamide denaturing gel.

METHODS. Radiolabelling and reaction processing were carried out as described for Fig. 3. R, *N*-cyanoimidazole. For strand sequences, see Fig. 2 legend. Lane 1, S_1^* ; lane 2, F_1^* . Each of the reaction mixtures (pH 6.15) for lanes 3–9 initially contained 100 mM imidazole-HCl, 5 μ M $ZnCl_2$, 100 mM NaCl, 10 mM *N*-cyanoimidazole, 1 μ M $S_1 \cdot S_2$ (except for lane 9), 2 μ M each of F_2 , F_1 , F_1 -mis-1 [5' P-TTCCTCCTCCCC 3'], F_1 -mis-2 [5' P-TTTCCTCCTCCCC 3'], F_1 -mis-3 [5' P-TTCCTCCTCCCC 3'] and F_1 -mis-4 [5' P-TCTCTCTCTCCTC 3'] in 100 μ l. Each reaction mixture contained only one radiolabelled sequence as follows. Lane 4, F_1^* ; lane 5, [F_1 -mis-1]*; lane 6, [F_1 -mis-2]*; lane 7, [F_1 -mis-3]*; lane 8, [F_1 -mis-4]*; lane 9, F_1^* ; lane 10–13 were same as in 5–8, respectively, except for the absence of F_1 . The reaction mixtures were incubated for 24 h at 20 °C.

and its components would be driven towards dissociation by excess fragments that would strongly bind the released single strand, and that the rate of ligation of the newly assembled duplex would be optimum¹⁸. Figure 3 depicts the experimental evidence for this constant-pH cycle. Thus pre-annealed ($S_1 \cdot S_2$) duplex (lacking 5' phosphates, to avoid end ligation) was incubated with a 1.2-fold excess of F_1^* and F_2 (lacking 5' phosphate) in the presence of *N*-cyanoimidazole to produce, in 85% yield, strand $F_1^* - F_2$ ($\equiv S_1^*$) (lane 6, Fig. 3). In the presence of F_1 , F_2 and a sixfold excess of F_3 (lacking 5' phosphate), and F_4^* , the same experiment demonstrated the formation of the second strand ($F_3 - F_4^*$) ($\equiv S_2^*$) in 70% overall yield (lane 8, Fig. 3). Appropriate control experiments confirmed the requirement for the template in this process (lanes 5 and 7, Fig. 3). Employing the above conditions, three successive replication cycles were demonstrated starting with 2.0 nmol of template ($S_1 \cdot S_2$) and purifying the duplex DNA between cycles using a centricon concentrator. The total amount of 24-monomer duplex DNA and the overall yield were determined both by counting radioactivity corresponding to incorporated and free fragments after electrophoresis, and by checking absorbance by ultraviolet-visible spectroscopy. The 24-monomer duplex DNA formed contained predominantly replicated template ($S_1 \cdot S_2$) but also small amounts of end-ligation products resulting from triple-helix formation as determined by control experiments. There is a roughly fivefold increase in the total amount of 24-monomer duplex DNA over three cycles ($\sim 75\%$ yield for each cycle). Whether this represents exponential growth is not yet clear, however—the precise kinetics and the scope and mechanism of the observed end ligation are under further investigation.

We have also examined selection effects and the fidelity of the replication process. Figure 4 summarizes several experiments in which four mismatched F_1 fragments (F_1 -mis) were allowed to compete with F_1 and among themselves. As seen from lanes 4–9 (Fig. 4), only F_1 participated predominantly in the cycle (lanes 5 and 6 showed small amounts of F_1 -mis-1 and F_1 -mis-2 incorporation respectively), reflecting lower tendencies of the mismatched sequences to form triple-helical structures with the template ($S_1 \cdot S_2$). It was noted, however, that in the absence of F_1 , the sequence with only one mismatch base (F_1 -mis-1 $\equiv 5'$ -P-TTCCTCCTCCCC 3'; mismatched base in bold) enters the replication cycle successfully (lane 10, Fig. 4). Thus, 'mutated' sequences can be replicated, albeit with lower efficiency.

The chemical processes described here demonstrate the viability of proliferation of duplex DNA by relying on the principles of complementarity involved in DNA triple- and double-helix formation and using simple chemical reactions and reagents. In the present example we made use of a template whose complement is identical to the template itself. In principle, however, this system could be extended to non-palindromic templates, provided that the appropriate substrate fragments are supplied to enable replication of both strands of the duplex. The possibility of extending these principles to the synthesis of more sophisticated DNA systems and other important biopolymers, including RNA, proteins and polysaccharides, is intriguing. The simplicity of the process may allow further improvements and automation, and the demonstrated selection and informational nature of the molecules involved may have significant implications for the origins of life on Earth^{19–23}. □

Received 11 February; accepted 4 March 1994.

- Orgel, L. E. *Nature* **358**, 203–209 (1992).
- Joyce, G. F. *Nature* **338**, 217–224 (1989).
- Hoffmann, S. *Angew. Chem. int. Edn engl.* **31**, 1013–1016 (1992).
- Tjivikua, T., Ballesten, P. & Rebek, J. Jr. *J. Am. chem. Soc.* **112**, 1249–1250 (1990).
- Feng, Q., Park, T. K. & Rebek, J. Jr. *Science* **256**, 1179–1180 (1992).
- von Kiedrowski, G. *Angew. Chem. int. Edn engl.* **25**, 932–935 (1986).
- von Kiedrowski, G., Wlotzka, B. & Helbing, J. *Angew. Chem. int. Edn engl.* **28**, 1235–1237 (1989).
- von Kiedrowski, G., Wlotzka, B., Helbing, J., Matzen, M. & Jordan, S. *Angew. Chem. int. Edn engl.* **30**, 423–426 (1991).
- Goodwin, J. T. & Lynn, D. G. *J. Am. chem. Soc.* **114**, 9197–9198 (1992).
- Kanavarioti, A. *J. theor. Biol.* **158**, 207–219 (1992).

11. Eschenmoser, A. & Lowenthal, E. *Chem. Soc. Rev.* **21**, 1–16 (1992).
12. Maher, L. J., Dervan, P. B. & Wold, B. J. *Biochemistry* **29**, 8820–8826 (1990).
13. Leubke, K. J. & Dervan, P. B. *J. Am. chem. Soc.* **111**, 8733–8735 (1989).
14. Inoue, T. & Orgel, L. E. *Science* **219**, 859–862 (1983).
15. Acevedo, O. L. & Orgel, L. E. *J. molec. Biol.* **197**, 187–193 (1987).
16. Chen, C. B., Inoue, T. & Orgel, L. E. *J. molec. Biol.* **181**, 271–279 (1985).
17. Kanavarioti, A., Bernasconi, C. F., Albers, D. J. & Baird, E. E. *J. Am. chem. Soc.* **115**, 8537–8546 (1993).
18. Kanaya, E. & Yanagawa, H. *Biochemistry* **25**, 7423–7430 (1986).
19. Miller, S. L. in *Cold Spring Harbor Symp. quant. Biol.* **18**, 17–27 (1987).
20. Gedulin, B. & Arrhenius, G. in *Early Life on Earth* (ed. Bengtson, S.) 91–110 (Nobel Symp. No. 84, Columbia Univ. Press, New York, 1993).
21. Ferris, J. P. & Ertem, G. J. *Am. chem. Soc.* **115**, 12270–12275 (1993).
22. Hunziker, J. et al. *Helv. chim. Acta* **76**, 259–352 (1993).
23. Eschenmoser, A. *Chem. Biol. Introd.* issue iv–v (1994).

ACKNOWLEDGEMENTS. We thank L. E. Orgel and G. F. Joyce for discussion. This work was supported by The Scripps Research Institute, the US NIH, Pfizer, Inc. and Glaxo Pharmaceuticals, Inc.

Self-replication of complementary nucleotide-based oligomers

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THE development of non-enzymatic self-replicating systems based on autocatalytic template-directed reactions is a current objective of bioorganic chemistry^{1–6}. Typically, a self-complementary template molecule AB is synthesized autocatalytically from two complementary template fragments A and B^{7–16}. Natural replication of nucleic acids, however, utilizes complementary rather than self-complementary strands. Here we report on a minimal implementation of this type of replication¹⁷ based on cross-catalytic template-directed syntheses of hexadeoxynucleotide derivatives from amino-trideoxynucleotides. In our experiments, two self-complementary and two complementary templates compete for their combinatorial synthesis from four common trimeric precursors. We provide kinetic evidence that cross-catalytic self-replication of complementary templates can proceed with an efficiency similar to that of autocatalytic self-replication of self-complementary templates. We observe selective stimulation of template synthesis, and thus information transfer, on seeding the reaction mixtures with one of four chemically labelled templates bearing the sequence of the reaction products. Our results bring a stage closer the development of schemes that might explain how replicating systems based on nucleic acids arose on the prebiotic Earth.

A minimal implementation of cross-catalytic self-replication is given by the general reactions (1) and (2) where A, B and AA, BB denote complementary template fragments and templates, respectively:



The reactions proceed via reversibly formed termolecular complexes (A·A·BB and B·B·AA) in which irreversible ligation of template fragments takes place. The resulting template duplex (AA·BB) reversibly dissociates to give the template molecules. As long as both templates are formed by the same type of chemistry, conditions that enable cross-catalytic formation of complementary products AA and BB will also allow for the autocatalytic synthesis of self-complementary products AB and BA:



Consequently, reactions (1)–(4) must be equally efficient for best observability of the cross-catalytically coupled reactions (1) and (2). Experiments on the template-directed synthesis of hexadeoxynucleotides from trimeric fragments revealed that the reactions are predominantly controlled by the stacking of nucleotide bases flanking the reaction site in the termolecular complex¹⁸. Thus in order to approach equal reaction efficiency, all products should have the same base sequence at the newly formed internucleotide link. The above general reaction model is realized in our example where the trideoxynucleotide sequences are CCG (A) and CGG (B).

Figure 1a–c shows the structures, the basic reactions and the template-catalysed condensations of the trimeric constituents chosen for the experiments. Two 5'-protected trideoxynucleotide 3'-phosphates, ^N₃CCGp (Ap, N₃ is 5'-azido) and ^{MTM}CCGp (Bp, MTM is 5'-methylthiomethoxy), were reacted with two 5'-aminotrideoxynucleotide 3'-(*o*-chlorophenyl)-phosphates, nCCGp^{CIPh} (nA) and nCCGp^{CIPh} (nB), in the presence of water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) as the condensing agent¹⁹. The formation of the four 3'-5'-phosphoramidate linked hexamers (ApnA, BpnB, ApnB and BpnA, respectively) resulting from combinatorial synthesis was monitored by high-performance liquid chromatography. A typical elution profile showing the composition of the reaction mixture after 1.5 h reaction time is depicted in Fig. 2; the experimental conditions are given in the legend. Figure 3a reflects the time-course of product formation. All four products are formed with comparable rates. In experiments in which similar hexamers (5'-azido-3'-(2-phenylthioethyl)-phosphates) were synthesized separately so that the cross-catalysis could not occur, the 1-h yields of the self-complementary products were five times higher than those of the complementary products.

The reactions were then followed in the presence of one of the hexadeoxynucleotide templates CCGCCG (ApA), CGGCCG (BpB), CCGCGG (ApB) and CGGCCG (BpA). These hexamers, bearing a central 3'-5'-phosphodiester link, can be considered as chemically labelled reaction products. They are eluted at earlier retention times in reversed phase chromatography because they lack lipophilic groups at their 5'- and 3'-ends. The template effects are, however, comparable to the respective products as follows from the results reported here. Figure 3b–e provides evidence that each hexamer template stimulates the formation of the one and only reaction product whose sequence is complementary to the respective template sequence. Sequence information is thus transferred from the template to its copy. The effect of a single complementary template, ApA or BpB, is more pronounced than the effect of a single self-complementary template. When both templates, ApA and BpB, are present (Fig. 3f), the effects are comparable to those of the self-complementary templates. Remarkably, the effects of ApA and BpB are similar, although the base compositions are different. Base composition proved to be most important in template-directed copolymerizations of nucleoside 5'-phospho-(2-methyl)-imidazolidines²⁰. In these pioneering attempts at non-enzymatic replication, in which monomers instead of oligomers were employed as informational units, only pyrimidine-rich templates were found to be efficient—purine-rich templates were not.

The experimental data from Fig. 3a–f can be rationalized using the following simplified reaction model where AA, BB, AB and BA are general species representing both externally added and internally synthesized hexamer templates:



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Equations (5)–(8) describe the hexamer-independent (uncatalysed) synthesis of the respective hexamers. Equations (9)–(12) comprise the reversible formation of the respective termolecular complex and the irreversible template-directed synthesis leading to the respective hexamer-duplex. Equations (13)–(15) deal with the reversible association of single-stranded hexamers to give the respective hexamer-duplexes. Activation of the 3'-phosphates **Ap** and **Bp** by EDC and the hydrolysis of the activated species **Ap*** and **Bp*** are not explicitly considered in the model. These processes are known to proceed as fast quasi-reversible reactions

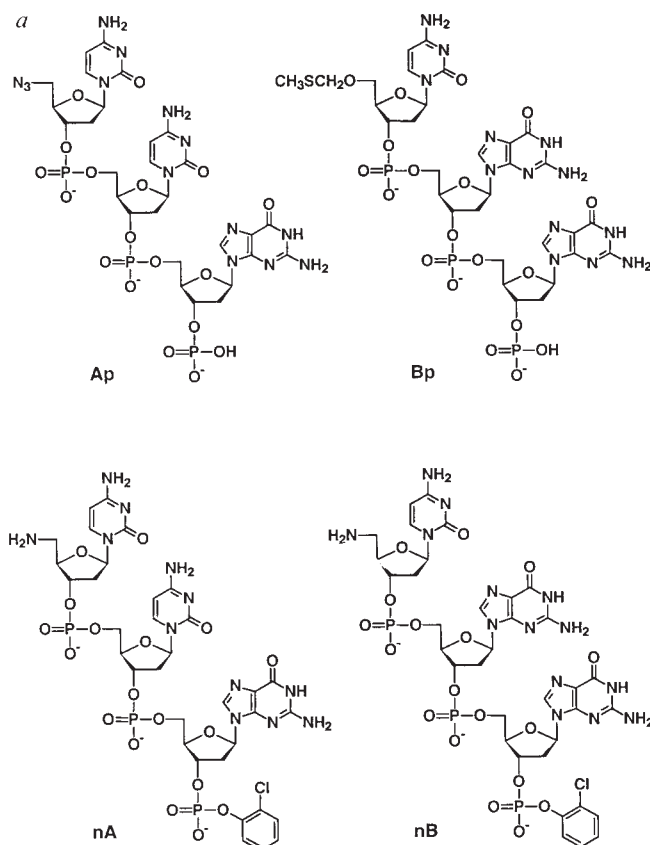
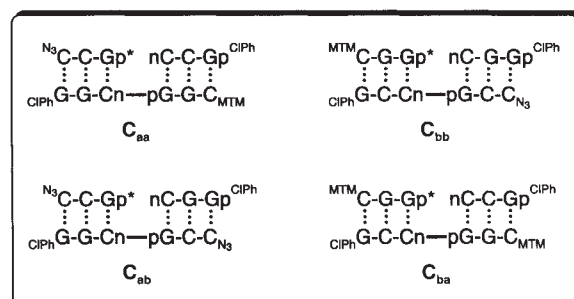
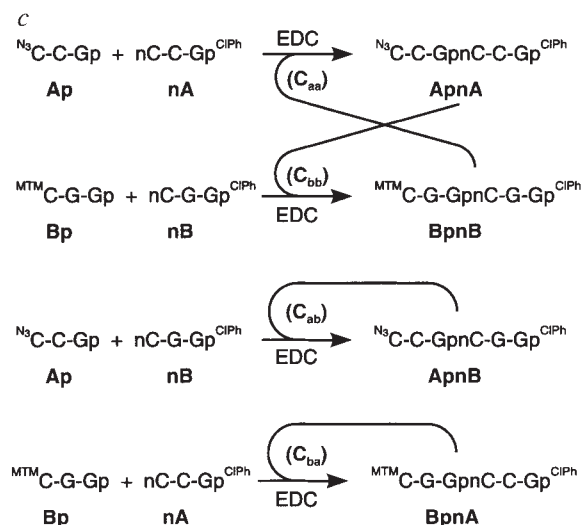
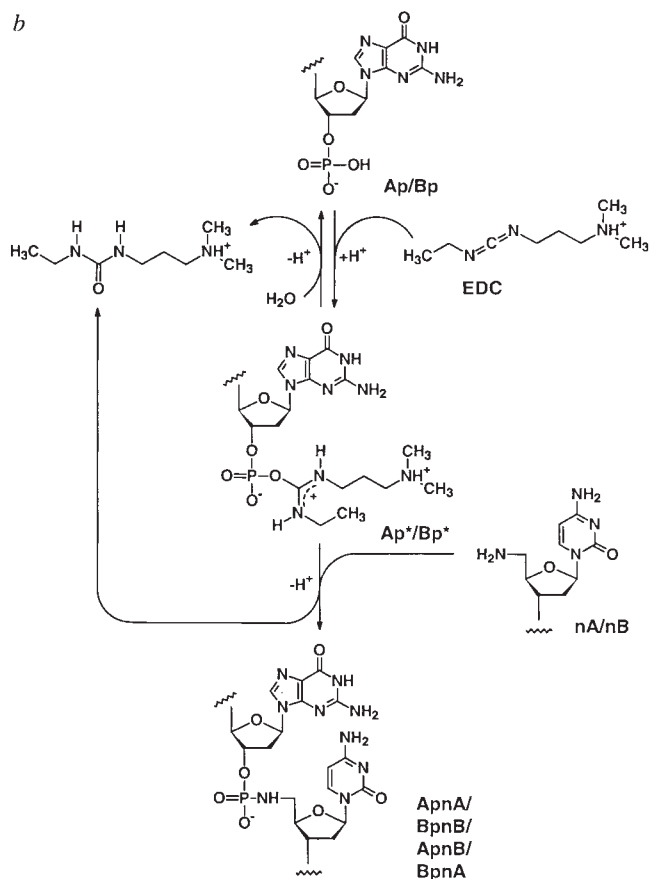


FIG. 1 a, Chemical structures of the trideoxynucleotide derivatives **Ap**, **Bp**, **nA** and **nB**. b, Basic reactions of **Ap**, **Bp**, **nA** and **nB** in the presence of water-soluble carbodiimide EDC. The 3'-phosphate groups of **Ap** and **Bp** add to the carbodiimide EDC to yield the isourea derivatives **Ap*** and **Bp***, respectively. The activated 3'-phosphates are hydrolysed to reproduce the original phosphates. Thus, activation and hydrolysis proceed as quasi-reversible reactions. Alternatively, **Ap*** and **Bp*** react with the 5'-aminogroup of **nA** and **nB** to give the four possible hexameric 3'-5'-phosphoramidates **ApnA**, **BpnB**, **ApnB** and **BpnA** by combinatorial synthesis. c, The cross-catalytic formation of **ApnA** and **BpnB**, and the autocatalytic synthesis of **ApnB** and **BpnA** proceed via termolecular complexes **C_{aa}**, **C_{bb}**, **C_{ab}** and **C_{ba}**, respectively. In these complexes, in which the reaction products serve as templates to bind the respective trideoxynucleotide precursors by Watson-Crick base pairing (dots between base symbols), the reactive phosphate- and amino-groups are oriented into close spatial proximity. Terminal groups are written as superscripts if the sequence is to be read in 5'-3'-direction. Subscripted symbols indicate the reverse direction.



establishing the stationary concentration of Ap^* and Bp^* within some minutes¹⁹. Furthermore, as EDC is present in 100-fold excess over the 3'-phosphates, the degree of activation of the latter is assumed to be time-independent over the first 90 min. The rate constants of reactions (5)–(12) are thus apparent constants which depend on the concentration of the carbodiimide.

The above model was employed to calculate the theoretical time courses shown in Fig. 3a–f using the computer program SimFit (available from G.v.K. on request). The experimental time-courses of all experiments were evaluated simultaneously to generate a single set of three apparent rate constants k_a , k_b and k_c , from which the theoretical time-courses for all experiments were derived. We assumed, equally efficient uncatalysed reactions (5)–(8) (k_a), equally efficient catalysed reactions (9)–(12) (k_b) and equally efficient duplex dissociations. The latter were characterized by the backward rate constants of reactions (13)–(15) (k_c) providing a symmetry factor of 2 for the self-complementary hexamers. The forward rate constants of reactions (13)–(15) can be chosen arbitrarily, so long as the irreversible reactions remain rate-determining (internal equilibrium). We used a value of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ for all hexamers in our calculation. From the resulting value of k_c it follows that the dissociation constant of the complexes is $\sim 10 \mu\text{M}$ at $T = 30^\circ\text{C}$,

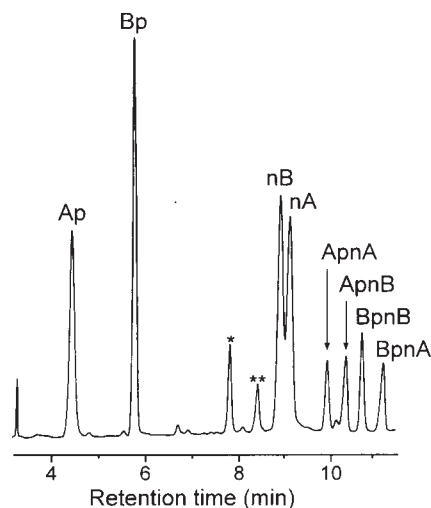


FIG. 2 High-performance liquid chromatography (HPLC) elution profile from the reaction between **Ap**, **Bp**, **nA**, **nB** and EDC after a reaction time of 90 min; ^{13}C CCG and ^{15}N CCG were used as standards, and are shown as * and ** on the elution profile. Reaction conditions and initial concentrations (indicated by subscript zero) as follows; $T = 30^\circ\text{C}$, $[\text{Ap}]_0 = [\text{Bp}]_0 = [\text{nA}]_0 = [\text{nB}]_0 = 1 \text{ mM}$, $[\text{EDC}]_0 = 0.2 \text{ M}$, $[\text{HEPES} (\text{Na}^+/\text{H}^+)] = 0.1 \text{ M}$, pH 7.55.

METHODS. HPLC: Column, Nucleosil 120-7 C_{18} (Macherey and Nagel); initial buffer (IB), 0.1 M aqueous NH_4HCO_3 ; final buffer (FB), acetonitrile/water 3:7 (v/v); flow rate: 1 ml min^{-1} ; gradient, 15% to 55% FB within 10 min, 55% to 75% FB within 3 min; detection, simultaneous monitoring of ultraviolet absorbance at wavelengths of 254 nm (above profile) and 273 nm; Equipment (Kontron), two pumps 420, autosampler 460, ultraviolet detector 430, data acquisition using system D450. For all reactions, a batch mixture of the four trideoxynucleotides in equimolar concentrations was prepared. Aliquots of this mixture were lyophilized in Eppendorf tubes using a SpeedVac evaporator (Savant). For experiments with templates, aliquots were co-lyophilized with the hexadeoxynucleotides. The reactions were started by the addition of $10 \mu\text{l}$ of a freshly prepared solution of EDC in HEPES buffer. The mixtures were then vortexed, centrifuged and drawn into a series of $1\text{-}\mu\text{l}$ precision capillaries (Hirschmann, non-heparinized). During a reaction, the capillaries were stored in small, air-tight containers under a water-saturated atmosphere. After the given interval of time the reaction was quenched by draining the capillaries into $250 \mu\text{l}$ of IB. HPLC injection volume was $150 \mu\text{l}$.

in good agreement with thermodynamic data on similar hexadeoxynucleotides¹⁸.

Remarkably, although several simplifications were introduced, and although data from six different experiments each with four observables were evaluated simultaneously, the above model (based on only three iterated rate constants) reproduces the experimental time courses quite well. Implicit in the model is that the cross-catalysis between complementary templates occurs with product inhibition as in the case of self-complementary templates. Product inhibition is the rationale for the so-called square-root law of template autocatalysis^{6,7} which does not allow

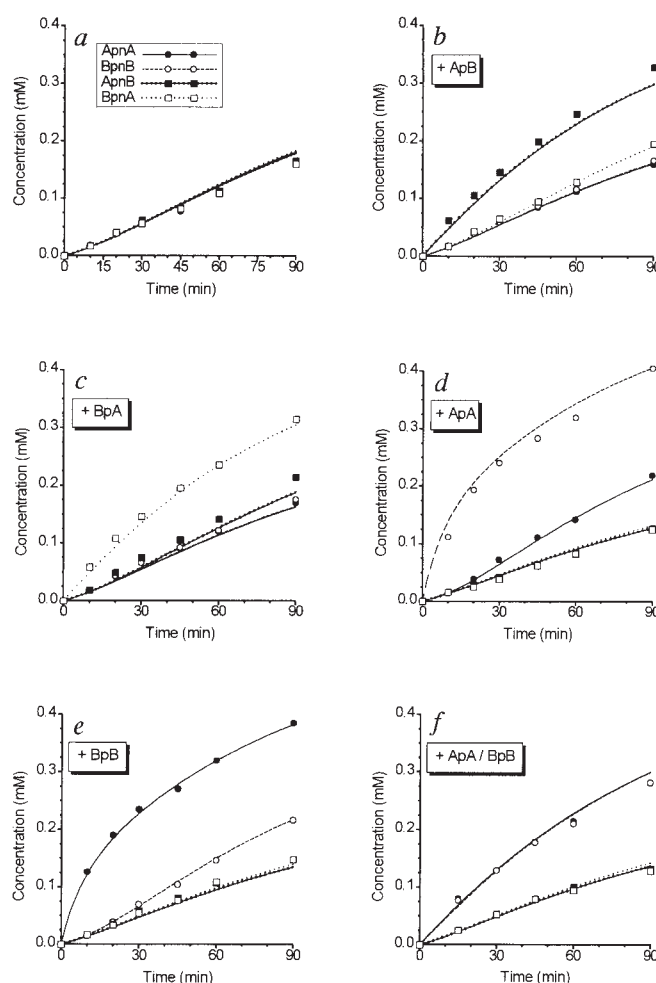


FIG. 3 Experimental points and theoretical curves describing the time courses of the formation of **ApnA**, **BpnB**, **ApnB** and **BpnA** in the absence (a), and in the presence of $\sim 0.16 \text{ mM}$ concentrations of hexadeoxynucleotide templates **ApB** (b), **BpA** (c), **ApA** (d), **BpB** (e) and **ApA + BpB** (f). In the calculations using the program SimFit, the initial concentrations of the model species were calculated from the respective HPLC peak areas using calibration data for each component. $[\text{AA}]_0$, $[\text{BB}]_0$, $[\text{AB}]_0$ and $[\text{BA}]_0$ were taken from the concentrations of **ApA**, **BpB**, **ApB** and **BpA**, respectively. All duplex concentrations were set to zero. Theoretical species concentrations were calculated by integration of the set of stiff rate equations that were derived from the model using SimFit. Theoretical values for the observable concentrations were calculated using the expressions: $[\text{ApnA}]_{\text{theor}} = [\text{AA}] + [\text{AA}\cdot\text{BB}] - [\text{AA}]_0$; $[\text{BpnB}]_{\text{theor}} = [\text{BB}] + [\text{AA}\cdot\text{BB}] - [\text{BB}]_0$; $[\text{ApnB}]_{\text{theor}} = [\text{AB}] + 2[\text{AB}\cdot\text{AB}] - [\text{AB}]_0$; $[\text{BpnA}]_{\text{theor}} = [\text{BA}] + 2[\text{BA}\cdot\text{BA}] - [\text{BA}]_0$. Nonlinear optimization was based on least-square approximations employing the Newton–Raphson algorithm. The resulting rate constants (see text) and error estimates were $k_a = (2.07 \pm 0.04) \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$, $k_b = (1.68 \pm 0.07) \times 10^{-3} \text{ M}^{-2} \text{ s}^{-1}$, $k_c = 8.98 \pm 0.81 \text{ s}^{-1}$. The off-diagonal elements of the covariance matrix were; $\text{cov}(k_a, k_b) = -0.368$, $\text{cov}(k_a, k_c) = +0.219$ and $\text{cov}(k_b, k_c) = -0.970$.

for exponential amplification of template concentration; in the best case, growth may be parabolic^{6,11}.

The evolutionary consequence of parabolic growth is coexistence of competing replicators²¹. They can coexist at stationary concentration levels whose ratio is given by the ratio of the square of the replication rate constants²¹. Non-replicating molecules that compete for their common precursors can reach only levels of concentration whose ratio is determined by the ratio of their formation rate constants, and not by the ratio of their squares²¹. Thus, parabolic growth is expected to enhance selectivity: small differences in reactivity should result in larger concentration differences as compared to those found during synthesis of non-autocatalytic molecules. On the other hand, selection—which is the characteristic outcome of competition between biological species—necessitates exponential growth²². In terms of growth laws and their evolutionary consequences, parabolic replicators are thus halfway between chemical and biological systems.

Our results may have important implications for theories of the origin of life, including those that invoke self-organization of complex reaction systems involving collective replication of oligonucleotides²³. Assuming that pathways for the formation of short random oligonucleotides from monomeric precursors (such as the clay-catalysed pathway²⁴) existed on the early Earth, it seems plausible that the first self-replicating systems used oligomers as informational units and operated on the basis of template-directed ligations. The recent observation of self-replication of double-helical DNA oligomers via the formation of triple-helical intermediates²⁵ also supports the view that non-

enzymatic self-replication of nucleic acids, based on autocatalytic and cross-catalytic ligations, may have been possible on the early Earth. □

Received 1 March; accepted 24 March 1994.

- Orgel, L. E. *Nature* **358**, 203–209 (1992).
- Hoffman, S. *Angew. Chem. int. Edn engl.* **31**, 1013–1016 (1992).
- Eschenmoser, A. & Loewenthal, E. *Chem. Soc. Rev.* **21**, 1–16 (1992).
- Bachmann, P. A., Luisi, P. L. & Lang, J. *Nature* **357**, 57–59 (1992).
- Famulok, J. S., Nowick, J. & Rebek, J. *Acta. chem. scand.* **46**, 315–324 (1992).
- von Kiedrowski, G. *Bioorg. Chem. Front.* **3**, 113–146 (1993).
- von Kiedrowski, G. *Angew. Chem. int. Edn engl.* **25**, 932–935 (1986).
- Zielinski, W. S. & Orgel, L. E. *Nature* **327**, 346–347 (1987).
- von Kiedrowski, G., Wlotzka, B. & Helbing, J. *Angew. Chem. int. Edn engl.* **28**, 1235–1237 (1989).
- Tjivikua, T., Ballester, P. & Rebek, J. *J. Am. chem. Soc.* **112**, 1249–1250 (1990).
- von Kiedrowski, G., Wlotzka, B., Helbing, J., Matzen, M. & Jordan, S. *Angew. Chem. int. Edn engl.* **30**, 423–426 and 892 (1991).
- Terfort, A. & von Kiedrowski, G. *Angew. Chem. int. Edn engl.* **31**, 654–656 (1992).
- Hong, J.-I., Feng, Q., Rotello, V. & Rebek, J. *Science* **255**, 848–850 (1992).
- Feng, Q., Park, T. K. & Rebek, J. *Science* **256**, 1179–1180 (1992).
- Böhler, C., Bannwarth, W., Luisi, P. L. *Helv. chim. Acta* **76**, 2313–2320 (1993).
- Achilles, T. & von Kiedrowski, G. *Angew. Chem. int. Edn engl.* **32**, 1198–1201 (1993).
- Kanavarioti, A. *J. Theor. Biol.* **158**, 207–219 (1992).
- Wlotzka, B. thesis, Univ. Göttingen (1992).
- Dolinnaya, N. G., Tsytoch, A. V., Sergeev, V. N., Oretskaya, T. S. & Shabarova, Z. A. *Nucleic Acids Res.* **19**, 3073–3080 (1991).
- Inoue, T. & Orgel, L. E. *Science* **219**, 859–862 (1983).
- Szathmáry, E. *Trends Ecol. Evol.* **6**, 366–370 (1991).
- Eigen, M. & Schuster, P. *The Hypercycle. A Principle of Natural Self-Organization* (Springer, Berlin, 1979).
- Kauffman, S. A. *The Origins of Order* (Oxford Univ. Press, New York, 1993).
- Ferris, J. P. & Ertem, G. J. *Am. chem. Soc.* **115**, 12270–12275 (1993).
- Li, T. & Nicolaou, K. C. *Nature* **369**, 218–221 (1994).

ACKNOWLEDGEMENTS. We thank E. Szathmáry and H.-J. Grützmacher for comments and suggestions on the manuscript. This work was supported by Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie and NATO.

Evidence for only minor contributions from bacteria to sedimentary organic carbon

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BECAUSE their molecular signatures are often prominent in extracts of sediments, bacteria are thought to be important contributors to petroleum source beds¹. It has been shown recently^{2,3}, however, that abundances of biomarkers do not always reflect relative contributions to sedimentary organic carbon (C_{org}). The contribution of photosynthetic green sulphur bacteria to sediments can be assessed effectively because the diagenetic products of distinctive carotenoids from these organisms occur widely^{4–11} and their biomass is isotopically labelled, being enriched in ^{13}C (refs 12, 13). We show here that, although sediments and oils from the Western Canada and Williston basins contain prominent biomarkers of photosynthetic bacteria, the absence of ^{13}C enrichment in the total C_{org} requires that the bacterial contribution is in fact minimal. Although the importance of bacterial reworking of sedimentary debris cannot be doubted¹⁴, we argue that our findings, when considered in conjunction with those from other settings, suggest that bacterial biomass may commonly represent only a minor component of total C_{org} in carbonaceous rocks.

The Western Canada and Williston basins contain source beds which have generated large quantities of oil and gas¹⁵. Remarkably, pyrolysates of the high-molecular-weight fractions of the Palaeozoic sediments and oils contain high abundances of 1,2,3,4-tetramethylbenzene (TMB, **I** in Fig. 1). This is most evident in immature sediments from the Upper Devonian Duvernay Formation, for which pyrolysates of kerogens, asphaltenes and polar fractions are dominated by **I** (Fig. 2a and b). Oils generated from these sediments also carry this signature: **I** is a clearly recognizable component of the oil (Fig. 2c) and is also dominant in its asphaltene pyrolysate (Fig. 2d). The abundance of **I** in pyrolysates has been attributed to the presence of macromolecularly bound aromatic carotenoids (**II–VI**) which can generate **I** by cleavage of the C–C bond β to the aromatic ring^{16–18}. However, unambiguous evidence for a relationship between **I** and bound carotenoids has been lacking. Here we use four lines of evidence to confirm this hypothesis, and discuss geological and palaeo-ecological implications of these findings.

First, octadecahydro diaromatic carotenoids are abundant components of the aromatic hydrocarbon fractions of the sediments and related oils, here unambiguously identified as isorenieratane (**VII**) and a novel C_{40} diaromatic component (**VIII**). Feasible precursors are, respectively, isorenieratene (**III**) and an unprecedented diaromatic carotenoid (**IX**) with a distinctive aromatic substitution pattern¹⁹. Second, the γ -cleavage products of the macromolecularly bound carotenoids in the pyrolysates are dominated by a 3:1 mixture of 1-ethyl-2,3,6- and 1-ethyl-3,4,5-trimethylbenzene (**X** and **XI**). This is consistent with incorporation of carotenoids **III** and **IX** in a ratio 1 to 1. Indeed, 1,2,3,5-TMB (**XII**), the presumed β -cleavage product of macromolecularly bound **VIII**, is the second most abundant C_4 -alkylated benzene in the pyrolysates and oils (Fig. 2). Third, selective cleavage of C–S bonds in macromolecular aggregates in the polar fraction using Raney-nickel-released **VII** and **VIII**, indicating that carotenoids **III** and **IX** (or their partially transformed counterparts) do occur in a sulphur-bound form. The total incorporation of bacterial carotenoids is even higher than that indicated by the selective cleavage, because pyrolysis of the residue indicated that only 10% of the bound carotenoids were

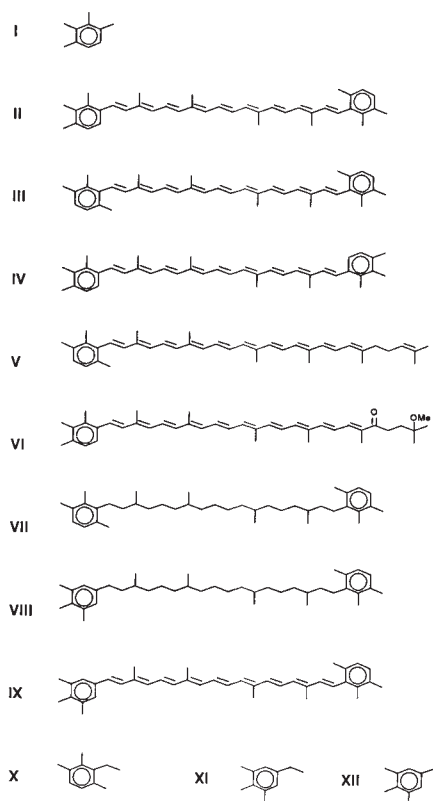


FIG. 1 Structures of compounds cited in the text. **I**, 1,2,3,4-tetramethylbenzene; **II**, renieratene; **III**, isorenieratene; **IV**, renierapurpurin; **V**, chlorobactene; **VI**, okenone; **VII**, octadecahydro-isorenieratene (isorenieratane); **VIII** and **IX**, a novel carotenoid and its partially hydrogenated derivative, respectively; **X**, 1-ethyl-2,3,6-trimethylbenzene; **XI**, 1-ethyl-3,4,5-trimethylbenzene; **XII**, 1,2,3,5-tetramethylbenzene.

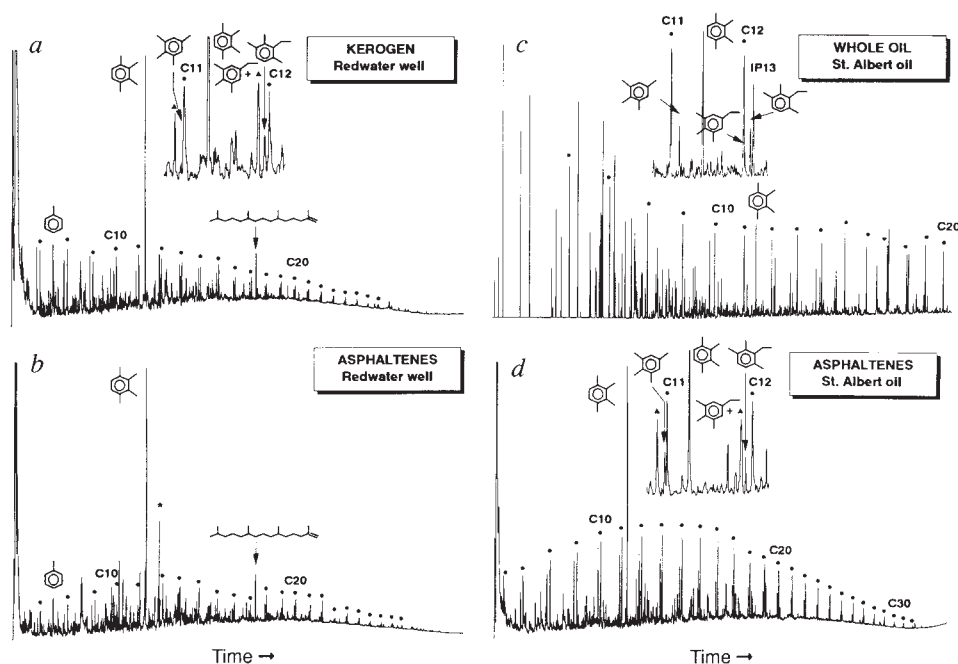
FIG. 2 Gas chromatograms (GC) of flash pyrolysates (Curie temperature 610 °C) of kerogen (a) and asphaltene (b) fractions of the immature Duvernay Formation (Redwater well, 1,159.9 m), and d, asphaltene fraction of the St. Albert oil. c, Partial whole-oil chromatogram (C₄ to C₂₀) of the same oil. Filled circles indicate *n*-alkanes, stars indicate 3-ethyl-4-methyl-1-H-pyrrole-2,5-dione, filled triangles indicate 1-dodecene. Numbers indicate total numbers of carbon atoms. Insets show enlarged areas of interest.

METHODS. The kerogen was isolated from the solvent-extracted rock sample through a series of treatments with hydrochloric and hydrofluoric acids¹⁸. Asphaltenes were precipitated from the extract (obtained by Soxhlet extraction with CH₃OH/CH₂Cl₂, 1/7) and oil using a 40-fold excess of *n*-heptane. On-line flash pyrolysis (Py) was performed using a FOM-4LX Curie point pyrolysis unit directly coupled to a Hewlett Packard 5890 Series II gas chromatograph fitted with a 25 m × 0.32 mm CP-Sil 5 (film thickness 0.12 μm) fused-silica capillary column, temperature programmed from 0 to 320 °C (hold time 10 min) at 3 °C min⁻¹. Structural assignments were made based on Py-GC-mass spectrometry (MS) data by comparison of GC and MS data with those of synthetic standards. MS conditions; VG-70S instrument, ionization

released by desulphurization. Fourth, the TMBs, ethyltrimethylbenzenes, free and S-bound **VII** and **VIII**, and previously identified^{9,18} aryl isoprenoids with the 2,3,6-substitution pattern from both the oil and sediment (Fig. 3) are enriched in ¹³C by 12–24‰ relative to other hydrocarbons and bulk fractions, indicating a distinct source for the diaromatic carotenoids.

These data are consistent with a depositional palaeoenvironment in which photosynthetic green sulphur bacteria (Chlorobiaceae) flourished⁹, a situation comparable with today's Black Sea^{4,10,20}. These bacteria contain isorenieratene (**III**) as a highly characteristic carotenoid²¹. Moreover, fixation of carbon proceeds by means of the reverse tricarboxylic acid cycle and leads to unusually high contents of ¹³C in the biosynthetic products^{12,13}. For example, in Miocene evaporites and recent sediments from the Black Sea, S-bound **VII** is enriched in ¹³C by ~14‰ relative to algal lipids^{10,11}, comparable to the enrichments of ¹³C in 1,2,3,4-TMB, 1-ethyl-2,3,6-trimethylbenzene, aryl isoprenoids, and S-bound **VII** relative to algal-derived hydrocarbons in both the Duvernay oil and sediment (Fig. 3). The additional enrichment of ¹³C in free **VII** (amounting overall to 24‰, see Fig. 3) is explained by a kinetically controlled fractionation occurring during incorporation of isorenieratene (**III**) into macromolecular organic matter. No such post-biosynthetic enrichment has been previously observed in sedimentary biomarkers, but is favoured in this case by two factors. First, quantification of the diagenetic products of the diaromatic carotenoids showed that free **VII** amounts to only 0.5% of the total isorenieratene carbon preserved in the sediment. It is, therefore, the residue of a carbon pool from which a 200-fold greater quantity of ¹³C-depleted material has been withdrawn, subjecting the residue, by difference, to dramatic enrichment. Second, and crucially, isotopic fractionation will occur at 18 of the 40 carbon positions within the molecule (that is, all of the sites at which C–S bond formation can occur). In these circumstances, only a modest kinetic isotope effect (0.6‰) is required to explain the observed enrichment.

The analogous distribution of components related to isorenieratane (**VII**) and **VIII** indicate that their carotenoid precursor



voltage 70 eV, mass-to-charge (*m/z*) range 40–800, cycle time 1.8 s. Whole oils were analysed using a 30 m × 0.25 mm DB-5 capillary column, temperature programmed from –60 to 200 °C at a rate of 2 °C min⁻¹.

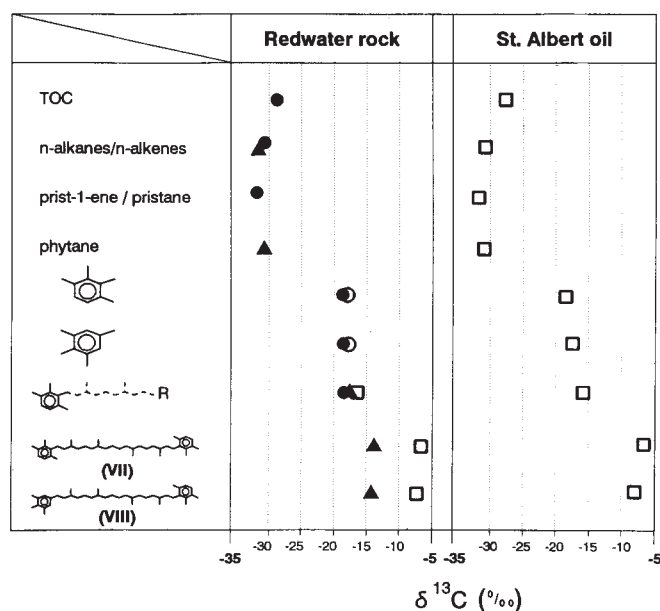


FIG. 3 Carbon isotopic compositions of total organic carbon (TOC) and selected hydrocarbons from all sedimentary fractions. Symbols used: ●, kerogen pyrolysate; ○, asphaltene pyrolysate; ▲, desulphurised polar fraction; □, hydrocarbon fraction. $\delta \equiv 10^3[(R_x - R_s)/R_s]$ in ‰, where $R \equiv {}^{13}\text{C}/{}^{12}\text{C}$, x designates sample, s designates the PDB standard, and $R_s = 0.0112$. Procedures for determination of δ for individual compounds have been reported elsewhere²⁵.

METHODS. Off-line pyrolysates (400 °C, 2 h) of kerogen and asphaltenes were trapped in hexane, separated by column chromatography into saturated and aromatic hydrocarbon fractions, and analysed by GC and GC-MS. Quantification was based on an internal standard, 1-phenyldodecane, added before pyrolysis. Sulphur-bound isorenieratane (VII) and the novel diaryl isoprenoid (VIII) were released by Raney nickel desulphurization of macromolecular aggregates in the polar fractions isolated from the extracts¹¹. Released hydrocarbons were separated by column chromatography and quantified by addition of 5-(1,1-d₂-hexadecyl)-2,3-dimethylthiophene before desulphurization. Free VII and VIII were concentrated and quantified¹⁸ in the aromatic hydrocarbon fraction of the sediment extract. The aryl isoprenoids and the components of the oil were concentrated using techniques reported elsewhere¹⁸.

sors were structurally related. Accordingly, IX is proposed as the precursor for VIII. The enrichment of ¹³C in the dia- and catagenetic products of IX (Fig. 3) suggests that it, too, derived from photosynthetic green sulphur bacteria, although IX has not been found in any contemporary organism. Possibly the species of Chlorobiaceae capable of biosynthesizing IX has become extinct, though the presence of VIII in seep oils sourced by Jurassic and Cretaceous sediments in the northern Gulf of Mexico (A.G.R., unpublished results) suggests that its occurrence extended into the Mesozoic era.

The structures of the diaromatic carotenoids favour formation of C-S bonds and thus incorporation in macromolecular fractions¹⁰. Preservation of these distinctive biomarkers can thus be particularly effective. Thermal cracking of macromolecular aggregates during burial will yield structurally characteristic TMBs and aryl isoprenoids. The prominence of these products in both the sediments (where they account for 1% of the C_{org}) and crude oils then suggests substantial contributions from green sulphur bacteria to the bulk of the organic carbon. Alternatively, it is possible that the aromatic carotenoids were selectively preserved, that the rest of the photosynthetic bacterial carbon was remineralized, and that—in spite of the prominence of the aryl isoprenoids—most of the C_{org} derives from other sources.

Isotope evidence allows a decisive choice between these alternatives. Specifically, the observed, 2.5‰ depletion of ¹³C in n-alkanes and n-alkenes relative to total organic carbon (TOC, Fig. 3) is typical of kerogens derived from communities in which

photosynthetic bacteria were absent²². If photosynthetic bacterial contributions to TOC had been significant, its δ value (see Fig. 3 legend) would have been elevated and the isotopic difference between it and the straight-chain hydrocarbons increased. Preservation of the 2.5‰ difference thus requires that photosynthetic bacterial contributions to TOC were inconsequential.

A reconsideration of previous results brings to light other cases in which, despite the prominence of bacterial biomarkers in sedimentary extracts, isotope evidence nevertheless indicates that bacterial contributions to total C_{org} were small. These are of particular significance because they involve other types of bacteria that are distributed even more widely than the green photosynthetic bacteria. In the Eocene Green River Shale, for example, isotopically distinct hopanes derived from methanotrophic and chemoautotrophic bacteria are prominent in extracts, but the bulk isotopic compositions are instead characteristic of oxygenic phytoplankton²³, requiring that contributions from these bacteria to total C_{org} were minor. In fact, significant portions of the kerogen are comprised of the resistant outer cell walls of the green microalga *Scenedesmus*²⁴. In the Eocene Messel Shale, ¹³C-depleted biomarkers²⁵ indicate that the depletion of ¹³C in kerogen relative to porphyrins and other algal products²⁶ could be partly due to bacterial contributions, notably from methanotrophic species. But resistant products of the green microalga *Tetradron minimum* are highly abundant²⁷ and apparently strongly depleted in ¹³C (ref. 28), thus providing a more likely cause for the depletion of ¹³C in TOC. A relatively minor impact of methane cycling on C_{org} is also suggested by the small contribution of methanogenic biomass to the Messel Shale, as revealed by the yield ($\leq 0.02\%$ of TOC) of archaeobacterial membrane lipids released from the kerogen²⁹. The different trends (with depth) in ¹³C contents of bacterially derived C₃₅ hopane and of TOC in the Monterey Formation³⁰ also indicate that in this open marine setting bacterial carbon has not contributed importantly to total C_{org}. Integration of all lines of evidence indicates that, in spite of the prominence of extractable bacterial biomarkers and in spite of the undoubted importance of bacterial reworking of sedimentary organic matter¹, net contributions of bacterial biomass are often small.

Clearly, the interpretive step leading from abundances of biomarkers to estimated sources of total C_{org} must be taken carefully. The instances of bias noted here involve sediments with relatively high contents of organic carbon, and separate considerations may apply to low-TOC sites, but the need for caution is clear. In all cases, the combination of microscopic and molecular studies³ should be helpful. □

Received 11 October 1993; accepted 31 March 1994.

- Ouirsson, G., Albrecht, P. & Rohmer, M. *Scient. Am.* **251**, 34–41 (1984).
- Kohnen, M. E. L., Sinninghe Damsté, J. S. & de Leeuw, J. W. *Nature* **349**, 775–778 (1991).
- Sinninghe Damsté, J. S., de las Heras, F. X. C., van Bergen, P. F. & de Leeuw, J. W. *Geochim. cosmochim. Acta* **57**, 389–415 (1993).
- Repeta, D. J. *Geochim. cosmochim. Acta* **57**, 4337–4342 (1993).
- Schwark, L. & Püttmann, W. *Org. Geochem.* **16**, 749–761 (1990).
- Xinke, Y., Pu, F. & Philp, R. P. *Org. Geochem.* **15**, 433–438 (1990).
- Ostroukhov, S. B., Arefev, O. A., Makushina, V. M., Zabrodina, M. N. & Petrov, A. A. *Neftekhimiya* **22**, 723–788 (1982).
- Schaeffé, J., Ludwig, B., Albrecht, P. & Ouirsson, G. *Tetrahedron Lett.* **41**, 3673–3676 (1977).
- Summons, R. E. & Powell, T. G. *Nature* **319**, 763–765 (1986).
- Sinninghe Damsté, J. S., Wakeham, S. G., Kohnen, M. E. L., Hayes, J. M. & de Leeuw, J. W. *Nature* **362**, 827–829 (1993).
- Kohnen, M. E. L. et al. *Science* **256**, 358–362 (1992).
- Quandt, I., Gottschalk, G., Ziegler, H. & Stichter, W. *FEMS Microbiol. Lett.* **1**, 125–128 (1977).
- Sirevag, R., Buchanan, B. B., Berry, J. A. & Troughton, J. H. *Arch. Microbiol.* **112**, 35–38 (1977).
- Parkes, R. J. et al. *Mar. Geol.* **113**, 55–66 (1993).
- Allan, J. & Creany, S. *Bull. Can. Petrol. Geol.* **39**, 107–122 (1991).
- Douglas, A. G., Sinninghe Damsté, J. S., Fowler, M. G., Eglinton, T. I. & de Leeuw, J. W. *Geochim. cosmochim. Acta* **55**, 275–291 (1991).
- Hartgers, W. A., Sinninghe Damsté, J. S. & de Leeuw, J. W. in *Organic Geochemistry—Advances and Applications in Energy and the Natural Environment* (ed. Manning, D. A. C.) 420–423 (Manchester Univ. Press, 1991).
- Requejo, A. G., Allan, J., Creany, S., Gray, N. R. & Cole, K. S. *Org. Geochem.* **19**, 245–264 (1992).
- Hartgers, W. A., Koopmans, M. P., Sinninghe Damsté, J. S. & de Leeuw, J. W. *J. chem. Soc., chem. Commun.* **23**, 1715–1716 (1993).

20. Repeta, D. J., Simpson, D. J., Jørgensen, B. B. & Jannasch, H. W. *Nature* **342**, 69–72 (1989).
21. Llaaen-Jensen, S. in *Photosynthetic Bacteria* (eds Clayton, R. K. & Sistrom, W. R.) 233–248 (Plenum, New York, 1978).
22. Eglinton, T. I., Fry, B. D., Freeman, K. H. & Hayes, J. M. in *Organic Geochemistry—Advances and Applications in Energy and the Natural Environment* (ed. Manning, D. A. C.) 411–416 (Manchester Univ. Press, 1991).
23. Collister, J. W., Summons, R. E., Lichtfouse, E. & Hayes, J. M. *Org. Geochem.* **19**, 265–276 (1992).
24. Derenne, S., Largeau, C., Berkalo, C. & Peniguel, G. in *Organic Geochemistry—Advances and Applications in Energy and the Natural Environment* (ed. Manning, D. A. C.) 408–411 (Manchester Univ. Press, 1991).
25. Freeman, K. H., Hayes, J. M., Trendel, J.-M. & Albrecht, P. *Nature* **343**, 254–256 (1990).
26. Hayes, J. M., Takigiku, R., Ocampo, R., Callot, H. J. & Albrecht, P. *Nature* **329**, 48–51 (1987).
27. Goth, K., de Leeuw, J. W., Püttmann, W. & Tegelaar, E. W. *Nature* **336**, 759–761 (1988).
28. Edlington, T. I. *Org. Geochem.* (in the press).
29. Chappe, B., Michaelis, W. & Albrecht, P. in *Advances in Organic Geochemistry* (eds Douglas, A. G. & Maxwell, J. R.) 265–274 (Pergamon, Oxford, 1979).
30. Schoell, M., Schouten, S., Sinnighe Damsté, J. S., de Leeuw, J. W. & Summons, R. *Science* **263**, 1122–1125 (1994).

ACKNOWLEDGEMENTS. We thank Exxon Production Research Company and Esso Resources Canada Ltd for the Duvernay samples. We also thank L. Yue, T. M. Xie and J. Primack for technical assistance. This work was partly supported by a PIONIER grant (J.S.S.D.) from the Netherlands Organization for Scientific Research (NWO) and, at Indiana University, by support from NASA.

Radar interferometric mapping of deformation in the year after the Landers earthquake

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ALTHOUGH the 1992 Landers, California, earthquake sequence occurred in an area well sampled by geodetic networks^{1–3}, the postseismic deformation in the months following the earthquake has been measured at only 15 geodetic stations⁴. Another shortcoming in the geodetic coverage occurs west of the primary rupture, where the existing geodetic observations suggest, but cannot resolve, sympathetic slip on secondary faults¹. Such measurements, which are needed to place the Landers earthquake sequence in the context of a recurring seismic cycle in California, can be obtained with the dense spatial coverage provided by satellite radar interferometry^{5–9}. Here we present radar maps of the surface deformation field which reveal features that would otherwise have been poorly sampled, particularly if the earthquake had occurred in a less accessible area. We see triggered slip at the level of several centimetres as far as 100 km along a complex fault system (Fig. 1). Field investigations¹⁰ show right-lateral slip reaching maxima of 4 m and 6 m, respectively 10 km and 40 km north of the main shock, in agreement with slip distributions estimated from geodetic and seismological data^{1–3,11–14}. The Landers event and the magnitude 6.2 Big Bear event on the same day are part of a sequence^{10,15} of earthquakes beginning with the magnitude (M_w) 6.1 Joshua Tree earthquake on 23 April 1992 and continuing through 1993. In the month following the Landers event, up to 6 cm of post-seismic displacement was observed near the fault¹. The magnitude of the post-seismic displacement decays exponentially with a characteristic time of ~48 days (ref. 4).

To measure the deformation, we construct interference patterns from the difference of two synthetic aperture radar (SAR) images acquired by the ERS-1 satellite. This new

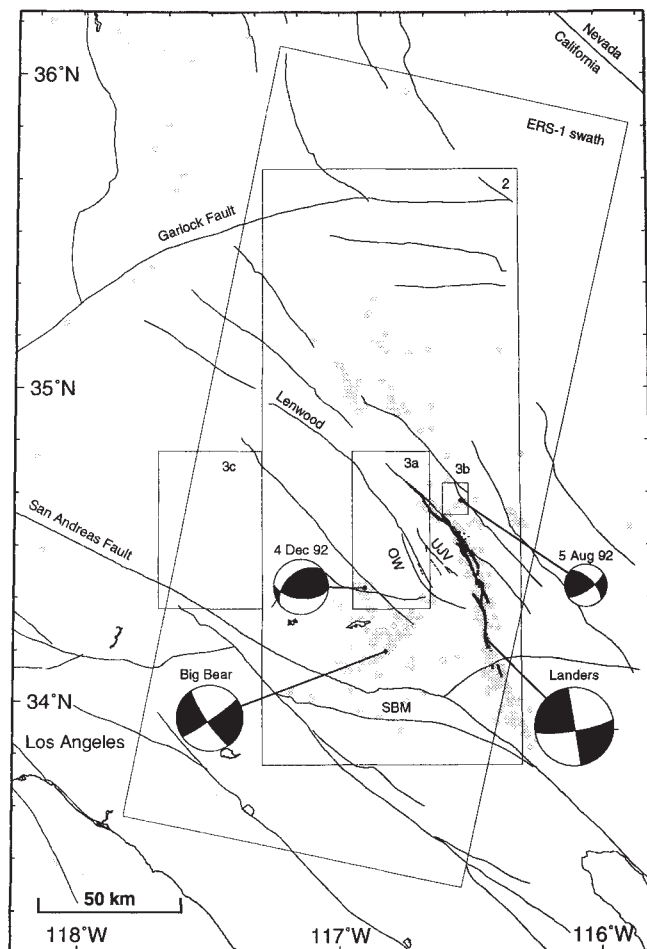


FIG. 1 Location map. Thin lines denote known faults²⁶ including the Old Woman (OW) and Upper Johnson Valley (UJV); heavy lines, the surface rupture mapped in the field¹⁰. Grey spots indicate the epicentres¹⁵ of earthquakes between 24 April and 30 December 1992. Focal mechanisms and epicentres¹⁵ are shown for the Landers ($M_w = 7.3$) and Big Bear ($M_w = 6.2$) earthquakes of 28 June 1992, as well as for the aftershocks of 4 December 1992 ($M_s = 5.1$) and 5 August 1992 ($M_s = 3.9$). Large, inclined box delimits the 120 × 275 km area covered by the ERS-1 satellite images. The radar antenna, at 785 km altitude, points across this swath at an azimuth of N77° W with an average angle of incidence of 23°. Numbered boxes delimit the regions shown in subsequent figures. The San Bernardino Mountains are denoted SBM.

geodetic technique can detect centimetre-sized changes in the ground surface^{5–9}. The resulting interferogram is a contour map of the change in range, that is the component of the displacement vector which points toward the satellite. The accuracy of the measurement is better than 4 cm in range, based on comparisons with ground surveys^{7,16}.

The techniques used to calculate the interferograms are essentially the same as described in our previous paper⁷. This approach requires only two images, but uses a digital elevation model¹⁷ to calculate the effect of topography. This is in contrast to the 'double-difference' technique, which can estimate topography directly, but requires at least three images^{6,9}. For this reason, our technique has a higher probability of finding enough suitable images. Even if an elevation model is unavailable, one can be produced by a variety of techniques, including interferometry using a pair of images free of crustal deformation. As in our earlier work, orbital effects are eliminated by adjusting the orbital parameters to minimize the fringes at the four corners of the interferogram. This procedure relies on the geophysical assumption of negligible deformation at distances greater than 100 km from the primary rupture.

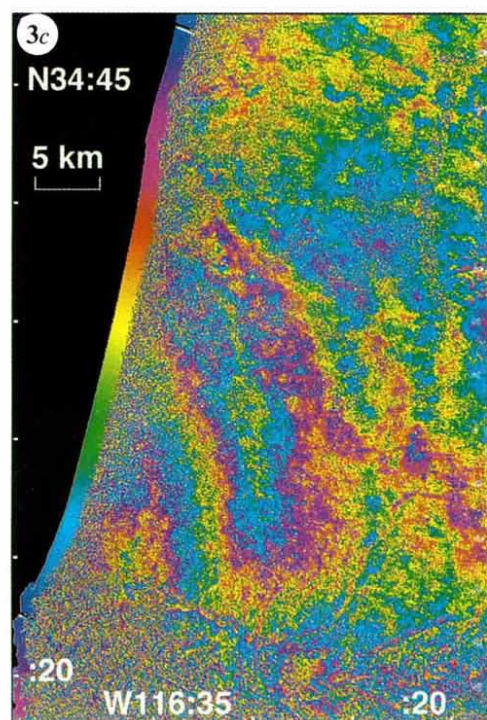
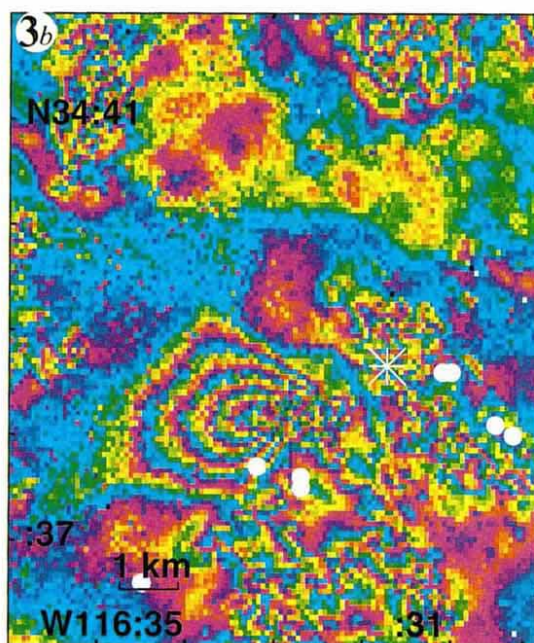
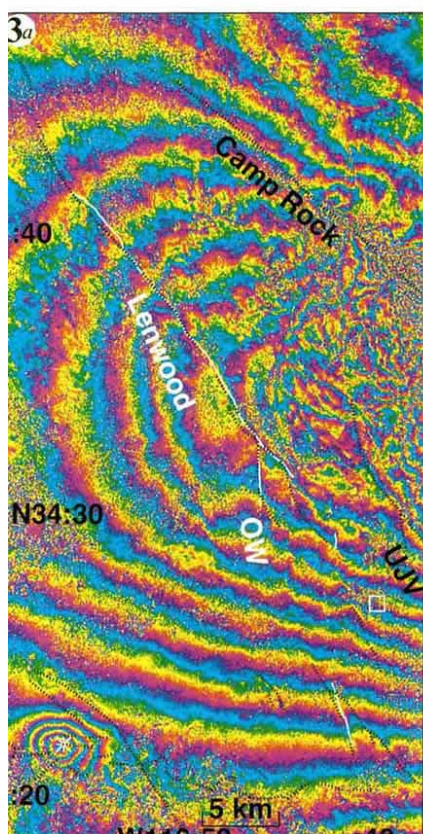
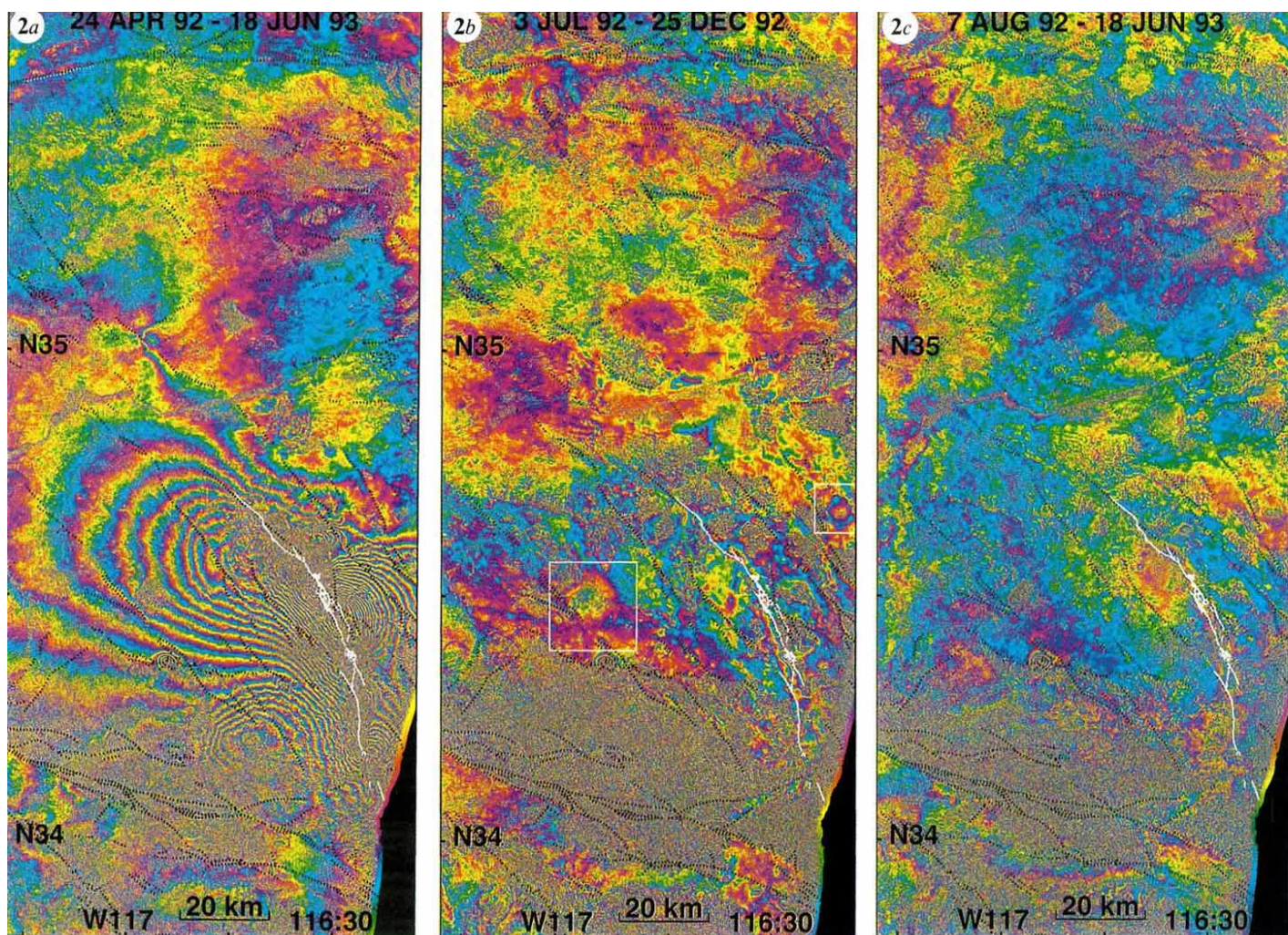


FIG. 2 Interferograms for the area delimited by the medium-sized box in Fig. 1. One cycle of colour corresponds to one interferometric fringe, equivalent to 28 mm of change in the range between the satellite and the reflector on the ground. Dotted black lines denote known faults²⁶, solid white lines, the surface rupture mapped in the field¹⁰. The quality of these interferograms is governed principally by the altitude of ambiguity for the pair of satellite orbits. This parameter is approximately inversely proportional to the horizontal distance between the satellite trajectories and represents the magnitude of the error in the digital elevation model necessary to create one fringe in the interferogram¹⁸. The elevation model was produced by the US Defense Mapping Agency which calls it a Digital Terrain Elevation Data, Level 1 (DTED-1), and is distributed by the US Geological Survey. The stated objective for its absolute vertical accuracy is ± 30 m, linear error at 90% probability¹⁷. An altitude of ambiguity several times larger than the 30-m uncertainty in the elevation model indicates that the interferometric map of the change in range is robust to errors in that model. *a*, Coseismic interferogram constructed from radar images acquired on 24 April 1992 and 18 June 1993. The altitude of ambiguity is 220 m, implying that a 30-m error in the elevation model would create an error of only 4 mm in the interferogram. This geometry is almost three times more favourable than the 80-m ambiguity for the first coseismic interferogram⁷. The improved coherence and precision reveal fringes which did not appear in the first coseismic interferogram near the San Bernardino Mountains. Here it is possible to count five closed fringes to the east of the Big Bear epicentre. The dotted white line in the northwest corner of the image is the segment of the Garlock fault inferred to have slipped. The successful correlation of two images separated by 14 months may be a fortunate consequence of the desert environment. We have also successfully spanned 1 year between two images of Mount Etna. We suspect that although correlation decays as a function of the time between images²⁷, it may reach an asymptote. *b*, Early postseismic interferogram constructed from radar images acquired on 3 July 1992 and 25 December 1992, which maps deformation in the period 5 to 180 days after the 28 June 1992 earthquake. The looping fringes near the rupture zone between its southern termination and $34^{\circ} 30'$ N indicate less than one cycle (~ 28 mm) of change in range during this time. The altitude of ambiguity for this interferogram is 55 m. *c*, Later postseismic interferogram constructed by differencing the two coseismic interferograms, the first composed of images acquired on 24 April 1992 and 7 August 1992 (ref. 7); the second composed of images acquired 24 April 1992 and 18 June 1993 (Fig. 2a). This map thus records deformation between 7 August 1992 and 18 June 1993, that is, between 40 and 355 days after the 28 June 1992 earthquake. The effective altitude of ambiguity of this composite interferogram is 130 m, which implies that a 30-m error in the elevation model would produce 6 mm of error in the interferogram. This value is close to the 7 mm precision estimated from the r.m.s. scatter, neglecting any deformation between 7 August 1992 and 18 June 1993. The two white squares shown in *b* enclose unidentified features. The western one, resembling a nut in a nutcracker, shows one complete cycle (28 mm) of negative change in range, indicating uplift. The eastern feature, resembling a rhomboid hazelnut, shows about half as much change in range. As neither of these features is visible in the second postseismic interferogram (*c*), they must have been generated between 3 July and 7 August 1992.

FIG. 3 *a*, Detail of the second coseismic interferogram (Fig. 2a). The four closed fringes around the epicentre (denoted by a star symbol) of the 4 December 1992 magnitude (M_L) 5.1 aftershock¹⁵ also appear in the two postseismic interferograms (Fig. 2b and c), but not in the first coseismic interferogram, for which the temporal coverage ended on 7 August 1992. Previously mapped faults²⁶ are shown by dotted black lines. The rupture of the Camp Rock fault segment mapped in the field¹⁰ is shown as a solid white line. Soggy Lake is denoted by a white square. Secondary faults with slip observed in the radar interferogram are shown by solid white lines. These include segments of the Garlock, Lenwood, Old Woman (OW) and possibly the Upper Johnson Valley (UJV) faults. To measure the offset on the faults, we 'binned' the phase measurements by printing only the three most significant bits in additional interferograms zoomed on the faults (not shown). These zoomed prints have eight levels of colour, and thus a resolution of $1/8$ cycle. The offset in range change $\Delta(\Delta\rho)$ is measured across the fault. The offset in the magnitude of the displacement vector $\Delta|\mathbf{s}|$ is estimated by assuming purely horizontal, right-lateral strike-slip displacement on the local trend of the fault. In this approximation, the ratio of $\Delta(\Delta\rho)$ to $\Delta|\mathbf{s}|$ equals the product $\sin\alpha \sin\phi$, where α is the angle between the fault and the satellite nadir ground track, and ϕ is the radar incidence

The ERS-1 satellite acquired one radar image of the epicentral area before the June 28 earthquake, and eight afterwards. Of the pairs spanning the earthquake, we have selected two on the basis of their altitude of ambiguity¹⁸ (Fig. 2). The first such coseismic interferogram includes the 40 days following the earthquake, and has been published previously⁷. The second (Fig. 2a) includes the interval extending to almost one year after the June 28 earthquakes. The 14 months between the pre-quake (14 April 1992) and post-quake (18 June 1993) images is a longer time lapse than any previous successful correlation.

For the interval from 5 to 180 days after the Landers earthquake, the map of range change is shown in Fig. 2b, which we call the 'early postseismic' interferogram. This map is expected to include 88% of the postseismic deformation, or 53 mm of displacement at most, if the 48-day exponential time constant is correct.

For the period from 40 to 355 days after the main shock, the deformation is measured by the difference of the two coseismic interferograms. This map, which we call the 'later postseismic' interferogram (Fig. 2c), includes less than 26 mm of displacement, according to the exponential model. Neglecting any intervening deformation, we infer the precision of the two coseismic interferograms to be 0.25 cycles (7 mm) in range from the r.m.s. scatter of their difference (Fig. 2c).

The coseismic interferograms (ref. 7 and Fig. 2a) are dominated by the signature of the earthquakes that occurred on 28 June. This large signal, creating over 20 cycles (560 mm) of change in range, has been modelled using dislocations in an elastic half space⁷. Inside the large, ear-shaped lobe in the centre of Fig. 2a, several linear 'tears' or discontinuities are apparent. These features are not artefacts. Three arguments indicate that they are generated by the earthquake sequence. First, they are common to both coseismic images. Their location and magnitude are identical, as evidenced by their absence from the difference (Fig. 2c). Second, these features are absent from the early post-seismic interferogram (Fig. 2b), which was processed using the same algorithm and elevation model as the coseismic interferograms. Third, if these features were artefacts of the elevation model, for example, we would expect their magnitudes to vary with the altitude of ambiguity¹⁸. They do not, as indicated by their disappearance in the difference (Fig. 2c).

These linear features are small offsets on secondary faults like those mapped in the field^{19,20}. On the Lenwood fault (Fig. 3a), we estimate $\sim 1/2$ cycle (14 mm) of change in range with an uncertainty of $\sim 1/8$ cycle (4 mm). Assuming the slip to be purely horizontal and parallel to the trace of the fault, we infer that the slip reaches a maximum of 51 ± 13 mm. On this fault, a separation of 45–50 mm was observed west of Soggy Lake (Fig. 3a), (G. Fuis, personal communication). The interferogram indicates that the slip continues both north on the Lenwood fault, and south onto the Old Woman fault (Fig. 3a).

On the Garlock fault, we measure $1/4 \pm 1/8$ cycle (7 ± 4 mm) of change in range, which implies 19 ± 10 mm of slip (Fig. 2a). This slip has not yet been confirmed in the field because the alignment arrays and triangulation networks have not been measured since 1987 (M. Clark, personal communication). Triggered slip on the Garlock fault is, however, predicted by models of loading during the Landers earthquake sequence^{21–23}.

angle. *b*, Detail of the early postseismic interferogram spanning the interval between 3 July and 25 December 1992. The concentric fringes are not visible in the later postseismic interferogram (Fig. 2c), constraining the date of this signal to fall between 3 July and 7 August 1992. The change in range is at least three fringes, or 84 mm. The star denotes the epicentre of the magnitude (M_L) 3.9 earthquake of 5 August 1992, a thrust mechanism at 7 km depth¹⁵. Other earthquakes with magnitude $3.0 < M_L < 3.5$ are shown by white circles. *c*, Detail of the early post-seismic interferogram spanning the interval between 3 July and 25 December 1992 showing a kidney-shaped feature with a change in range of ~ 1 cycle, or 28 mm. It is probably an atmospheric effect, as described in the text.

The date of the observed faulting must fall between the acquisition dates of the two images used for the first coseismic interferogram: 24 April and 7 August 1992. The field mapping performed on 1 July at Soggy Lake suggests that most of the secondary faulting occurred during, or soon after, the main shock.

There are two round features in the interferograms. The western one appears in Fig. 2a–c, but not in the first coseismic interferogram⁷. By elimination, we infer that it was generated between 7 August and 25 December, 1992. We interpret this feature as the displacement due to the magnitude (M_L) 5.1 aftershock¹⁵ of 4 December 1992. The feature consists of four closed concentric fringes, corresponding to 112 mm of change in range (Fig. 3a). This value is consistent with an elastic dislocation model²⁴ using 40 cm of slip on a 3×3 km patch of the north-dipping fault plane in a thrust focal mechanism¹⁵.

The second, eastern round feature (enlarged in Fig. 3b) exhibits three closed fringes with the opposite sign, indicating ~84 mm of downwelling. As it appears in the early postseismic interferogram (Fig. 2b), but not the later one (Fig. 2c), this feature must have been generated between 3 July and 7 August 1992. The largest nearby earthquake occurred on 5 August 1992, but it is apparently not responsible for the downwelling in light of its thrust mechanism, 7 km depth, and small 3.9 (M_L) magnitude¹⁵. The deformation may instead be due to the combination of several smaller ($M_L = 3.2$ – 3.3) and shallower (4–6 km) earthquakes (white circles in Fig. 3b) or possibly to subsidence.

The early postseismic interferogram (Fig. 2b) for the 5 months following the main shock is difficult to interpret because the magnitude of the deformation is over one hundred times smaller than the coseismic signal. We recognize several anomalous features with ~0.5–1.0 cycles (14–28 mm) of range change. The largest (25 km long) has a kidney shape and exhibits at least one closed fringe (Fig. 3c). This feature does not appear in the later postseismic interferogram. By elimination, it must have been generated between 3 July and 7 August 1992 if it is crustal deformation. This signal is not associated with an earthquake¹⁵ with magnitude greater than 3, or with subsidence from known areas of heavy water withdrawal (T. Holzer, personal communication). Nor is it visible in the early coseismic interferogram, even after subtracting a model for the June 28 deformation⁷. The most likely cause is thus an atmospheric propagation effect on 3 July or 25 December 1992.

Near the rupture zone, looping fringes are visible in the earlier postseismic interferogram (Fig. 2b) but not the later one (Fig. 2c), suggesting that most of the postseismic deformation occurred within 40 days of the main shock. These fringes span less than one complete cycle in the near field and even less in the rest of the early postseismic interferogram. This observation indicates that the postseismic range change in the first 6 months was less than 28 mm (Fig. 2b). This small signal is consistent with the range change of less than 0.3 cycles (8 mm) expected from the sum of postseismic slip with an exponential decay time of 48 days (ref. 4) and interseismic elastic strain accumulation over 6 months²⁵. From this consistency, we infer that an exponential decay time of 48 days is reasonable, and that quick afterslip, rather than slow lithospheric relaxation, is responsible for the postseismic deformation observed in Fig. 2b. □

12. Freymueller, J., King, N. E. & Segall, P. *Bull. seism. Soc. Am.* (in the press).
13. Hudnut, K. W. et al. *Bull. seism. Soc. Am.* (in the press).
14. Wald, D. J. & Heaton, T. H. *Bull. seism. Soc. Am.* (in the press).
15. Hauksson, E., Jones, L. M., Hutton, K. & Eberhart-Phillips, D. *J. geophys. Res.* **98**, 19835–19858 (1993).
16. Peltzer, G., Feigl, K. L. & Massonnet, D. *EOS (suppl., Oct. 26)* **74**, 60 (1993).
17. US Geological Survey *Digital Elevations Models: Data Users Guide* Vol. 5 1–34 (US Govt Print. Off., Washington DC, 1993).
18. Massonnet, D. & Rabaute, T. *IEEE Trans. Geosci. Remote Sensing* **31**, 455–464 (1993).
19. US Geological Survey Staff *EOS (suppl. Oct. 27)* **73**, 357–358 (1992).
20. Padgett, D. & Rockwell, T. *EOS (suppl. Oct. 26)* **74**, 68 (1993).
21. Harris, R. A. & Simpson, R. W. *Nature* **360**, 251–254 (1992).
22. Jaumé, S. C. & Sykes, L. R. *Science* **258**, 1325–1328 (1992).
23. Stein, R. S., King, G. C. P. & Lin, J. *Science* **258**, 1328–1332 (1992).
24. Okada, Y. *Bull. seism. Soc. Am.* **75**, 1135–1154 (1985).
25. Feigl, K. L. et al. *J. geophys. Res.* **98**, 21677–21712 (1993).
26. California Division Mines and Geology. *Preliminary Fault Activity Map of California* (Calif. Dept. of Conservation, Sacramento, 1992).
27. Zebker, H. A. & Villasenor, J. *IEEE Trans. Geosci. Remote Sensing* **30**, 950–959 (1992).

ACKNOWLEDGEMENTS. We thank R. Stein, M. Murray, J. Savage and D. Ponti for encouraging us to pursue the secondary faulting. E. Hauksson provided focal mechanisms.

Regulation of NMDA receptors in cultured hippocampal neurons by protein phosphatases 1 and 2A

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PHOSPHORYLATION of glutamate receptors is probably an important mechanism for modulating excitatory transmission^{1–4}. However, there is little direct evidence to indicate which protein phosphatases can dephosphorylate glutamate⁵ or other ligand-gated channels⁶, although it is known that protein phosphatases 1 and 2A play a major part in modulating voltage^{7–10} and second-messenger-gated channels¹¹. Here we report that in cultured hippocampal neurons, the *N*-methyl-D-aspartate (NMDA) receptor can be regulated by endogenous and exogenous serine/threonine protein phosphatases. Phosphatase inhibitors enhanced NMDA currents recorded using the perforated patch technique¹³ or in cell-attached patches, whereas protein phosphatases 1 or 2A decreased the open probability of these channels in inside-out patches.

To investigate the regulation of NMDA receptors by endogenous protein phosphatases (PP), we used calyculin A, a potent and specific inhibitor of PPI and 2A (refs 13–15). In some cases okadaic acid was also used¹⁶. We tested NMDA currents in the presence of high concentrations of its co-agonist glycine¹⁷: under these conditions the NMDA responses showed limited desensitization (Fig. 1a). Peak NMDA currents were potentiated ($47.9 \pm 4.6\%$ s.e.m.; $n = 8$) following the application of calyculin A (Fig. 1a, b). Results were the same with okadaic acid (125 nM–1 μ M), NMDA currents being enhanced by an average of $35.9 \pm 5.5\%$ ($n = 9$; results not shown). NMDA dose-response curves before and after treatment with calyculin A showed that potentiation of NMDA currents was associated with an increase in the maximal response to NMDA (Fig. 1b).

We next examined the dependence of this potentiation upon the concentration of added glycine. With no added glycine, NMDA currents were smaller and underwent desensitization¹⁸ (Fig. 2a). We could record NMDA currents in the absence of added glycine because our perfusing solutions probably contained glycine at nanomolar concentrations¹⁷. Calyculin A increased the initial peak (49.1 ± 6.8 ; $n = 10$) (Fig. 2a) of the NMDA current, but failed to alter the steady-state current recorded near the end of the agonist application (Fig. 2a, b).

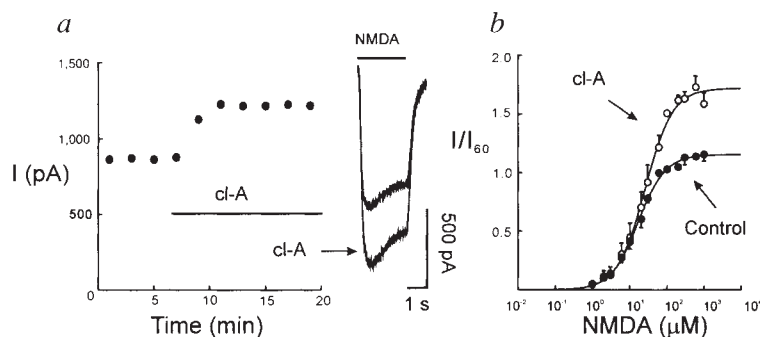
Received 6 December 1993; accepted 6 April 1994.

1. Murray, M. H., Savage, J. C., Lisowski, M. & Gross, W. K. *Geophys. Res. Lett.* **20**, 623–626 (1993).
2. Blewitt, G. et al. *Nature* **361**, 340–342 (1993).
3. Bock, Y. et al. *Nature* **361**, 337–340 (1993).
4. Shen, Z., Jackson, D., Feng, Y., Kim, M. & Cline, M. *Bull. seism. Soc. Am.* (in the press).
5. Massonnet, D. *Etude de Principe d'une Détection de Mouvements Tectoniques par Radar* (Centre National d'Etudes Spatiales, Toulouse, 1985).
6. Gabriel, A. K., Goldstein, R. M. & Zebker, H. A. *J. geophys. Res.* **94**, 9183–9191 (1989).
7. Massonnet, D. et al. *Nature* **364**, 138–142 (1993).
8. Goldstein, R. M., Engelhardt, H., Kamb, B. & Frollich, R. M. *Science* **262**, 1525–1530 (1993).
9. Zebker, H. A., Rosen, P., Goldstein, R. M., Gabriel, A. & Werner, C. L. *J. geophys. Res.* (submitted).
10. Sieh, K. et al. *Science* **260**, 171–176 (1993).
11. Cohee, B. P. & Beroza, G. C. *Bull. seism. Soc. Am.* (in the press).

FIG. 1 Calyculin A (cl-A) potentiates NMDA currents recorded using the perforated patch technique. *a*, Repeated responses were evoked by a rapid application of NMDA (500 μ M) in the presence of near-saturating concentrations of glycine (1 μ M) before and during the application of calyculin A (20 nM). *b*, NMDA dose-response curves were constructed (glycine 1 μ M) before and following calyculin A (20 nM). Paired dose-response curves ($n=4$) were normalized to the control current amplitude at 60 μ M (I_{60}) and fitted using the logistic equation¹⁸. Peak amplitudes are plotted. Calyculin A increased the maximal response (control, 1.15 ± 0.04 ; calyculin A 1.72 ± 0.12 ; $P < 0.05$; paired *t*-test). Calyculin A also increased the occurrence of spontaneous miniature synaptic currents (not shown) and a tonic release of presynaptic glutamate may have acted by a glycine-insensitive mechanism to predesensitize responses to applied NMDA. In the synaptic cleft, the concentrations of synaptically released glutamate exceed saturating values for NMDA receptors²⁴ and therefore phosphatase inhibition would be anticipated to enhance postsynaptic currents mediated by NMDA. Holding potential in this and other figures was -60 mV unless indicated. METHODS. Conventional monolayer cultures of hippocampal neurons were used for patch-clamp recording as described⁵. For perforated patch recordings, electrodes were first filled with (in mM) 120 CsCl, 35 CsOH, 10 HEPES, 2 MgCl_2 , 11 EGTA, 2 tetraethylammonium,

Thus the ratio of peak to steady-state current was increased by calyculin A and the time constant of desensitization was decreased (Fig. 2*b*). We therefore examined the relationship between added glycine concentration and the ratio of peak to steady-state currents evoked by a given concentration of NMDA. Calyculin A potentiated peak currents at all concentrations of glycine, whereas the enhancement of steady-state currents was dependent upon the concentration of added glycine. Thus the ratio of peak to steady-state currents decreased towards a value of one as the concentration of glycine was increased to almost saturating concentrations (Fig. 2*c*).

NMDA single-channel currents were recorded in the cell-attached configuration and in the presence of glycine to characterize further the actions of calyculin A. NMDA activated a



1 CaCl_2 , pH 7.3, and then back-filled with the same solution containing nystatin ($125 \mu\text{g ml}^{-1}$). The extracellular solution contained (in mM) 140 NaCl, 1.3 CaCl_2 , 5.4 KCl, 25 HEPES, 33 glucose and 0.001 tetrodotoxin, pH 7.4; 320–335 mosM. Amino acids were applied through a multi-barrel fast-perfusion system²⁵. Phosphatase inhibitors (LC Service, and Moana BioProducts, Honolulu) were directly perfused onto the cell through the control barrel.

channel of ~ 40 pS (Fig. 3*c*). Applications of calyculin A (20 nM) increased the average duration of channel opening by $62 \pm 21\%$ (Fig. 3*a, b*) and increased the frequency of channel opening (Fig. 3*b*) by $43 \pm 11\%$. In each of the patches ($n=4$), there was an increase in channel open probability (average, $204 \pm 92\%$). In contrast, the single-channel conductance was not affected by calyculin A (control 39.2 ± 1.2 pS; calyculin A 41.2 ± 1.4 pS).

We then tested the effects of PP1 and PP2A on channels recorded in inside-out patches. Applications of either protein phosphatase reduced the probability of NMDA channels opening (Fig. 3*d*). Concurrent applications of okadaic acid (10 nM) attenuated the action of PP2A but not PP1 ($n=2$), but at a higher concentration (100 nM) it prevented the depression evoked by each enzyme. The actions of both phosphatases were at least partially reversible suggesting that some protein kinase activity was still associated with the patches.

Our results show that NMDA receptors are regulated in hippocampal neurons by an endogenous protein phosphatase(s) that is identical to or is closely related to PP2A and/or PP1. This conclusion is in keeping with the presence of both of these phosphatases within the central nervous system¹⁹ and with the

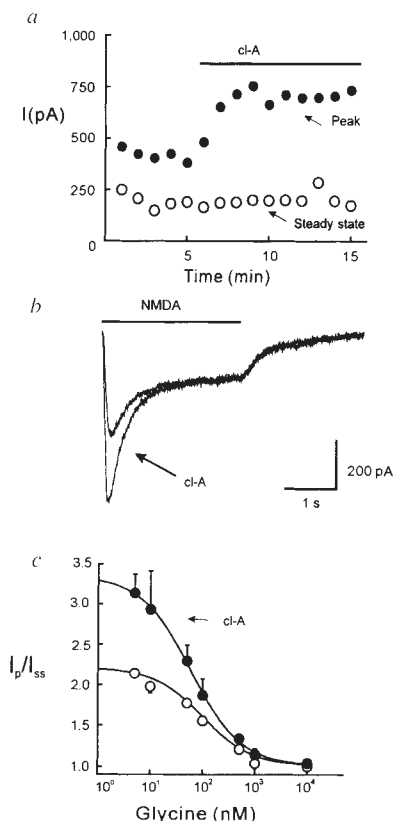
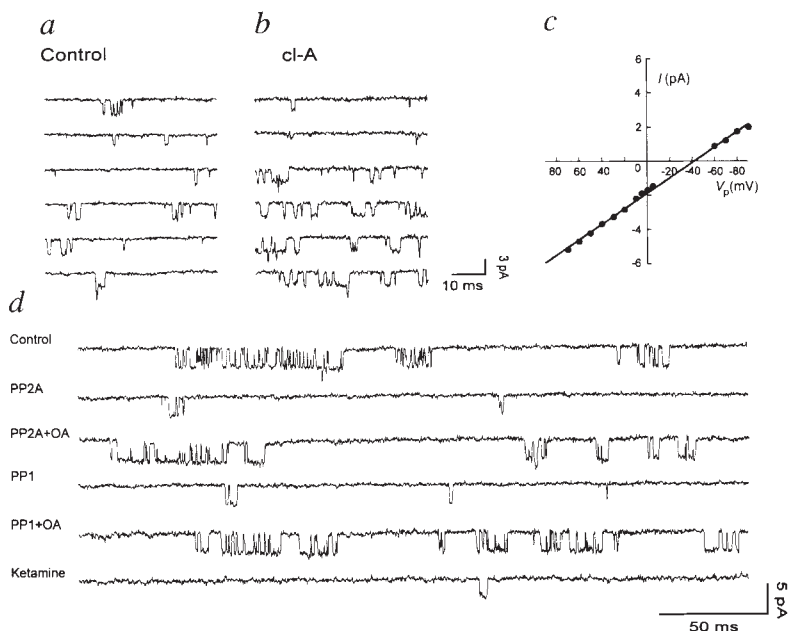


FIG. 2 The effect of calyculin A (cl-A) on NMDA-activated currents with respect to glycine concentration. *a*, In the absence of added glycine, calyculin A (20 nM) increases peak amplitude (●) without affecting steady-state (○) NMDA (500 μ M) currents. *b*, Representative current traces before and after application of calyculin are shown; each trace was the average of five consecutive responses. The rate of desensitization (τ , time constant) was estimated from a single exponential fit to the decay phase of NMDA currents. The time constant in this cell was decreased by calyculin A from 432 to 351 ms. In a series of cells ($n=5$), τ was reduced from an average of 440 ± 11 ms to 360 ± 9 ms following calyculin A ($P < 0.05$ paired *t*-test). *c*, A single dose of NMDA (500 μ M) was used to evoke currents in the presence of various concentrations of glycine (5 nM to 10 μ M). The ratio of peak/steady-state current was plotted against glycine concentration. Individual dose-response curves were fitted using the logistic equation in the form: $R = R_{\text{max}} / (1 + (d/EC_{50})^{nH}) + C$, where R represents the calculated ratio of peak/steady-state current at any given glycine concentration (d), R_{max} is the maximal ratio, EC_{50} is the concentration of glycine that produces 50% block of NMDA-current desensitization, nH is the estimated Hill coefficient, and C is a constant. Paired dose-response curves recorded in three cells were averaged before and after application of calyculin A (20 nM); calculated values are:

	R_{max}	nH	EC_{50} (nM)
Control	2.1 ± 0.1	-1.23 ± 0.05	116 ± 5.6
Calyculin A	$3.3 \pm 0.4^*$	-1.34 ± 0.26	$76.8 \pm 11.6^* (P < 0.05)$

FIG. 3 Modulation of NMDA single-channel activity by the phosphatase inhibitors calyculin A (cl-A) and okadaic acid (OA) and by the phosphatases PP1 and PP2A. A representative example of single-channel NMDA activity in the cell-attached configuration before (a) and after (b) the application of calyculin A (20 nM) is shown. The pipette potential (V_p) was +20 mV; NMDA (10 μ M) and glycine (1 μ M) were included in the recording pipette. The current-voltage relationship recorded from another patch is given in c. Calyculin A caused an increase in the probability of channel opening (0.053 to 0.1781), as well as an increase in mean open time (1.36 to 3.15 ms). An increase in open probability was also observed in an additional three patches (controls, 0.024, 0.085, 0.002; calyculin A, 0.033, 0.164, 0.011, respectively). The size of this increase was unexpected considering the small effects at low concentrations of agonist when the perforated patch technique was used; differences in the two techniques could have contributed to this discrepancy. Open probability (P_o) was calculated as described²⁶ from records of 51.2 to 153.6 seconds in duration. In d, a series of recordings are shown from a single inside-out patch, with the pipette held at +85 mV. Applications of PP2A (0.4 U ml⁻¹) or PP1 (1.2 U ml⁻¹) were associated with a reduction in channel open probability and these effects were attenuated by co-applications of 10 and 100 nM okadaic acid, respectively (control, 0.0235; PP2A, 0.0148; PP2A + OA, 0.0222; PP1, 0.0146; PP1 + OA, 0.0212). Results were similar in several additional patches (control, 0.025 ± 0.014 ; PP1, $0.6-1.2$ U ml⁻¹: 0.017 ± 0.008 , $n=5$; control, 0.043 ± 0.024 ; PP2A, $0.4-0.8$ U ml⁻¹: 0.028 ± 0.020 , $n=6$; Wilcoxon signed rank test one-tailed, $P < 0.05$). Bath application of ketamine (10 μ M) substantially reduced channel activity in this patch.

METHODS. Electrodes were filled with (in mM) 70 NaCl, 70 Na₂SO₄, 10 HEPES, 1.3 CaCl₂, 5 Cs₂SO₄, 33 glucose, 0.001 glycine and 0.01 NMDA, pH 7.4. In some cases, NaCl and Na₂SO₄ were replaced by Cs₂SO₄. Channels were identified both on the basis of single-channel conductance as well as sensitivity to blockade by ketamine²⁷. The cyto-



plasmic face of inside-out patches was perfused with the extracellular solution already described but also containing 10 mM EGTA (no added calcium) and 2 mM Mg-ATP. Single-channel data were sampled at 100 μ s and filtered at 2 kHz. The detection threshold for transitions between open and closure of the channels was 50% crossing, and channel openings that lasted < 300 μ s were not included in the analysis. Protein phosphatases were prepared as described^{28,29}. PP2A was isolated as a heterodimer that includes the 36K catalytic subunit and the 60K regulatory subunit; the PP1 preparation contains the catalytic fragment.

observation that PP1 is the principal protein phosphatase associated with postsynaptic densities²⁰ and therefore with glutamate receptors. Calyculin A and okadaic acid, at concentrations known selectively to inhibit PP1 and/or PP2A but not PP2B or PP2C (ref. 16), caused enhancement of both whole-cell NMDA as well as single-channel currents. Direct application of these phosphatases to inside-out patches indicated that the exogenous enzymes can decrease the activity of NMDA channels.

The binding of NMDA allosterically reduces the affinity of the receptor for glycine¹⁸. Therefore at low concentrations of glycine, the rate of desensitization is determined largely by the rate of dissociation of glycine, whereas at high concentrations the rate of binding of glycine is relatively greater than its rate of dissociation and there is therefore little desensitization¹⁸. Our evidence suggests that calyculin A increases the affinity of the receptor for glycine. At high concentrations of glycine, both peak and steady state currents are potentiated to the same degree. In contrast, when no glycine was added only the peak current appeared to be potentiated. The reason that we failed to resolve a potentiation of steady-state NMDA currents was probably due to the low availability of glycine in the solution.

There is strong evidence that NMDA receptors are directly phosphorylated by serine/threonine kinases⁴. Our results demonstrate that serine/threonine phosphatases also modulate the activity of this ligand-gated channel. The phosphatases may directly dephosphorylate these receptors, or alternatively they may target substrates that could themselves in turn regulate NMDA receptors. For example, cytoskeletal elements, including actin filaments, may modulate the activity of NMDA channels²¹, and protein phosphatases are essential for the maintenance and structural integrity of several major cytoskeletal components²². Moreover, protein phosphatases can themselves be regulated by endogenous inhibitors²³. Therefore the action of protein phos-

phatases, as well as their inhibitors, is a potentially important way to regulate NMDA receptors and excitatory synaptic transmission in the central nervous system. □

Received 10 August 1993; accepted 23 February 1994.

1. Raymond, L. A., Blackstone, C. D. & Huganir, R. L. *Trends Neurosci.* **16**, 147-153 (1993).
2. Ben-Ari, Y., Aniksztejn, L. & Bregestovski, P. *Trends Neurosci.* **15**, 333-339 (1992).
3. Swope, S. L., Moss, S. J., Blackstone, C. D. & Huganir, R. L. *FASEB J.* **6**, 2514-2523 (1992).
4. Tingley, W. G., Roche, K. W., Thompson, A. K. & Huganir, R. L. *Nature* **364**, 70-73 (1993).
5. Wang, L.-Y., Salter, M. W. & MacDonald, J. F. *Science* **253**, 1132-1135 (1991).
6. Mei, L. & Huganir, R. L. *J. Biol. Chem.* **266**, 16063-16072 (1991).
7. Chung, S., Reinhart, P. H., Martin, B. L., Brautigan, D. & Levitan, I. B. *Science* **253**, 560-562 (1991).
8. White, R. E., Schonbrunn, A. & Armstrong, D. L. *Nature* **351**, 570-573 (1991).
9. Reinhart, P. H., Chung, S., Martin, B. L., Brautigan, D. & Levitan, I. B. *J. Neurosci.* **11**, 1627-1635 (1991).
10. White, R. E., Lee, A. B., Shcherbatko, A. D. & Armstrong, D. L. *Nature* **361**, 263-266 (1993).
11. Gordon, S. E., Brautigan, D. L. & Zimmerman, A. L. *Neuron* **9**, 739-748 (1992).
12. Horn, R. & Marty, A. *J. Gen. Physiol.* **92**, 145-159 (1988).
13. Ishihara, H. et al. *Biochem. biophys. Res. Commun.* **159**, 871-877 (1989).
14. Kateo, Y., Fusetani, N., Matsunaga, S. & Hashimoto, S. *J. Am. Chem. Soc.* **108**, 2780-2781 (1986).
15. Suganuma, M. et al. *Cancer Res.* **50**, 3521-3525 (1990).
16. Cohen, P., Holmes, C. F. B. & Tsukitani, Y. *Trends biochem. Sci.* **15**, 98-102 (1990).
17. Johnson, P. W. & Ascher, P. *J. Physiol., Lond.* **455**, 339-365 (1992).
18. Benveniste, M., Clements, J., Vyklícký, L. & Mayer, M. L. *J. Physiol., Lond.* **428**, 333-357 (1990).
19. Nairn, A. C. & Shenolikar, S. *Curr. Opin. Neurobiol.* **2**, 296-301 (1992).
20. Cohen, P. *Rev. Biochem.* **58**, 453-508 (1989).
21. Rosenmund, C. & Westbrook, G. L. *Neuron* **10**, 805-814 (1993).
22. Eriksson, J. E. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 11093-11097 (1992).
23. Huang, F. L. & Glimmann, W. H. *Eur. J. Biochem.* **70**, 419-426 (1976).
24. Clements, J. D. et al. *Science* **258**, 1498-1501 (1992).
25. Johnson, J. W. & Ascher, P. *Nature* **325**, 529-531 (1987).
26. Oh, Y. & Benos, D. J. *Am. J. Physiol.* **264**, C1489-C1499 (1993).
27. Orser, B. A. & MacDonald, J. F. *Can. J. Anaesth.* **40**, A61 (1993).
28. Chen, J., Martin, B. L. & Brautigan, D. L. *Science* **257**, 1261-1264 (1992).
29. Brautigan, D. L., Shriner, C. L. & Gruppiso, P. A. *J. Biol. Chem.* **260**, 4295-4302 (1985).

ACKNOWLEDGEMENTS. We thank L. Brandes for technical assistance and M. W. Salter for comments on the manuscript. This work was supported by grants to the MRC of Canada group 'Nerve Cells and Synapses'. D.L.B. was supported in part by a grant from the American Cancer Society.

Regulation of NMDA receptors by tyrosine kinases and phosphatases

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PROTEIN-TYROSINE kinases (PTKs) and protein-tyrosine phosphatases (PTPs) are key enzymes in signal-transduction pathways for a wide range of cellular processes^{1,2}. PTKs and PTPs are highly expressed in the central nervous system³, which is consistent with the importance of tyrosine phosphorylation in neuronal function⁴⁻⁶. Protein phosphorylation is known to be involved in the regulation of neurotransmitter receptors^{7,8}, but the effects of tyrosine phosphorylation on neurotransmitter receptor function in the central nervous system are unknown. Here we present evidence that in mammalian central neurons tyrosine phosphorylation regulates the function of the NMDA (*N*-methyl-D-aspartate) receptor, a subtype of excitatory amino-acid receptor^{9,10}. NMDA-receptor-mediated whole-cell currents and intracellular Ca^{2+} responses are depressed by inhibition of PTKs. Conversely, NMDA currents are potentiated by intracellular application of the well characterized PTK $\text{pp60}^{\text{c-src}}$. NMDA currents are also potentiated by intracellular administration of an inhibitor of PTPs. Protein-tyrosine phosphorylation is a new mechanism for regulating NMDA receptors and may be important in neuronal development, plasticity and toxicity.

We used the whole-cell perforated-patch technique to record currents from spinal dorsal horn neurons. NMDA-receptor-

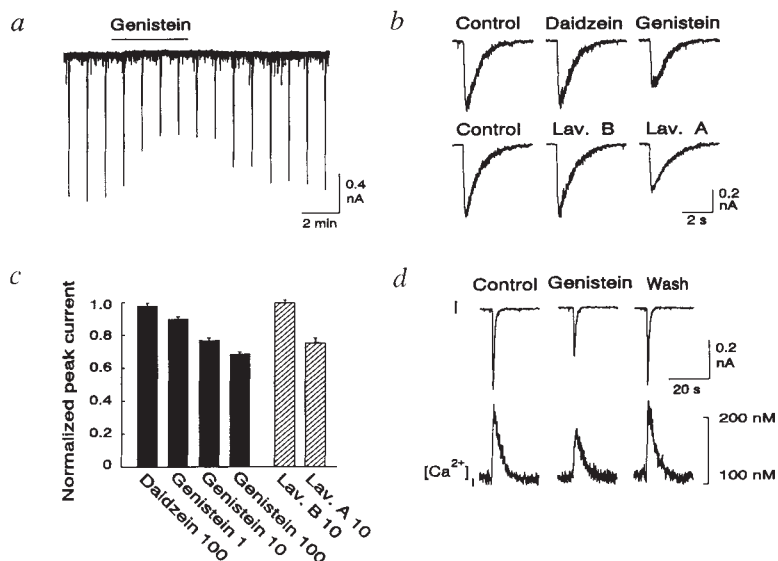
mediated responses were evoked by applying the selective NMDA receptor agonist, L-aspartate¹¹, using pressure from a micropipette near the neuron. Bath application of the PTK inhibitor genistein^{5,12} caused a progressive reduction of NMDA currents (Fig. 1a). After genistein was washed out, NMDA currents gradually recovered, indicating that the depression was reversible. Daidzein, an analogue of genistein that lacks PTK inhibitory activity¹², had no effect on NMDA currents (Fig. 1b). Depression of NMDA currents by genistein was concentration-dependent (Fig. 1c); the maximum depression was to $68 \pm 2\%$ (mean $\pm$ s.e.m.; $n = 10$ neurons) of the pre-application level and the estimated half-maximum concentration was $4 \mu\text{M}$. We also tested a structurally distinct PTK inhibitor, lavendustin A^{5,13}, and its inactive analogue, lavendustin B¹³. Lavendustin A ($10 \mu\text{M}$) reversibly depressed NMDA currents to $74 \pm 3\%$ control ($n = 5$ neurons), but they were unaffected by lavendustin B ($10 \mu\text{M}$; Fig. 1b, c).

An important consequence of activating NMDA receptors is a rise in the intracellular concentration of free Ca^{2+} ($[\text{Ca}^{2+}]_i$)^{9,10}. We therefore tested the effects of inhibiting PTKs on NMDA-receptor-mediated Ca^{2+} responses in neurons in which we measured $[\text{Ca}^{2+}]_i$ with the fluorescent dye Fura-2 and simultaneously recorded whole-cell currents. Application of genistein ($100 \mu\text{M}$) caused a reversible reduction in the amplitude of the Ca^{2+} responses while producing no change in the baseline level of $[\text{Ca}^{2+}]_i$ (Fig. 1d). The peak of the Ca^{2+} response was reduced to $61 \pm 8\%$ of control ($n = 5$ cells), similar to the reduction in the peak of the NMDA currents ($68 \pm 4\%$ of control). The depression of NMDA currents and Ca^{2+} responses by PTK inhibitors suggests that NMDA receptors are regulated by one, or possibly more, endogenous PTKs.

Because PTK inhibitors depressed NMDA currents, we used $\text{pp60}^{\text{c-src}}$, a well characterized PTK¹⁴, to test whether PTK could enhance the currents. We applied $\text{pp60}^{\text{c-src}}$ directly into the cells by means of diffusional exchange during standard whole-cell patch-clamp recording. Application of $\text{pp60}^{\text{c-src}}$ potentiated

FIG. 1 Inhibitors of protein-tyrosine kinases attenuate NMDA-receptor-mediated responses. Recordings were made using the perforated patch technique²¹. a, A continuous record of whole-cell current shows inward NMDA currents evoked repeatedly at 1-min intervals by pressure applications of L-aspartate ($250 \mu\text{M}$, 100 ms). Genistein ($100 \mu\text{M}$) was applied in the bathing solution over the period indicated by the bar. The trace begins 35 min after seal formation, at which time the patch perforation had been stable for 10 min. The membrane potential was held at -60 mV in this and all subsequent figures unless otherwise indicated. b, Upper records show representative individual NMDA currents before drug application ('control') and in the presence of $100 \mu\text{M}$ daidzein or $100 \mu\text{M}$ genistein; lower records are from another neuron and were taken before drug application, in the presence of $10 \mu\text{M}$ lavendustin B ('Lav. B') or $10 \mu\text{M}$ lavendustin A ('Lav. A'). c, Histogram showing effects of tyrosine kinase inhibitors and corresponding inactive analogues on normalized peak currents. Drug concentrations are indicated in μM . NMDA currents were normalized for each neuron by dividing the amplitude of the current in the presence of the drug by that of the control current before drug application. Data are mean normalized currents $\pm$ s.e.m. Numbers of neurons: 5 for daidzein; 5, 4 and 10 for the three concentrations of genistein, respectively; 4 for lavendustin B and 5 for lavendustin A. d, Traces of $[\text{Ca}^{2+}]_i$ are shown below corresponding whole-cell currents (I) recorded simultaneously. Individual responses recorded before ('control'), during ('genistein') and 10 min after ('wash') bath application of $100 \mu\text{M}$ genistein are shown.

METHODS. Spinal dorsal horn neurons in primary culture were prepared from fetal Wistar rats²². For recordings the cultures were bathed in an extracellular solution composed of (in mM): NaCl (140), KCl (5.4), HEPES (25), CaCl_2 (1.3), glucose (33), glycine (0.001) and tetrodotoxin (0.001), pH 7.35; osmolality, 310–320 mosM. The tip of the recording pipette was filled with a solution containing (in mM): CsCl (140), HEPES (10),



BAPTA (10), pH 7.25; osmolality, 300–315 mosM. The remainder of the pipette was then filled with a solution that also contained nystatin ($100 \mu\text{g ml}^{-1}$). Perforation of the patch usually stabilized within 20 min with series resistance of 15–20 M Ω . Intracellular Ca^{2+} concentration was measured with the Ca^{2+} -sensitive fluorescent dye Fura-2 in neurons that were subjected simultaneously to voltage clamp²². Sodium L-aspartate dissolved in extracellular solution was applied by pressure (150 kPa for 100 s) from a micropipette with its tip located 20–30 μm from the cell. Protein-tyrosine kinase inhibitors and control compounds were from LC Labs, Woburn, MA.

NMDA currents and a steady-state level was reached within 10 min after the start of recording (Fig. 2). At steady state, the amplitude of the NMDA currents was about twice that of the initial current ($n=9$ cells). In contrast, NMDA currents were not potentiated when pp60^{c-src} was removed by immunoprecipitation before recording ($n=4$ cells), when boiled pp60^{c-src} was applied intracellularly ($n=5$ cells), or when pp60^{c-src} was not included in the intracellular solution ($n=9$ cells). These results indicate that PTK activity causes potentiation of the currents. Further, currents were depressed by genistein to $57 \pm 2\%$ of pre-application level in cells treated with pp60^{c-src} (Fig. 2c; $n=6$), as opposed to $69 \pm 3\%$ in cells in which the kinase had not been included in the intracellular solution ($n=7$; $P < 0.01$, Student's t -test).

To confirm that the potentiated currents were mediated by NMDA receptors, we investigated the effects of the NMDA channel blocker, Mg²⁺, after the currents had reached a stable level. Extracellularly applied Mg²⁺ (500 μ M) caused a voltage-dependent blockade of the currents (data not shown). Further-

more, currents evoked by applying NMDA itself were potentiated by pp60^{c-src} in both dorsal horn neurons tested. NMDA-activated currents were also found to be potentiated by pp60^{c-src} perfused into hippocampal neurons ($n=2$; Fig. 2d). This observation indicates further that the potentiation is not unique to NMDA receptors in dorsal horn neurons.

To determine whether potentiation of these currents was due to a change in driving force or to a change in conductance, we examined the current-voltage (I - V) relationship. After potentiation had been established, the reversal potential of the currents was -5 to 0 mV (Fig. 2c). This range of reversal potentials was similar to that of NMDA currents recorded in neurons without pp60^{c-src}. Application of genistein did not cause a change in the reversal potential of the potentiated currents but produced a decrease in the slope of the current-voltage curve (Fig. 2c). Taken together, these observations indicate that PTK-induced potentiation of currents was due to an increase in NMDA-receptor-activated conductance.

The possibility that NMDA currents are regulated by PTPs

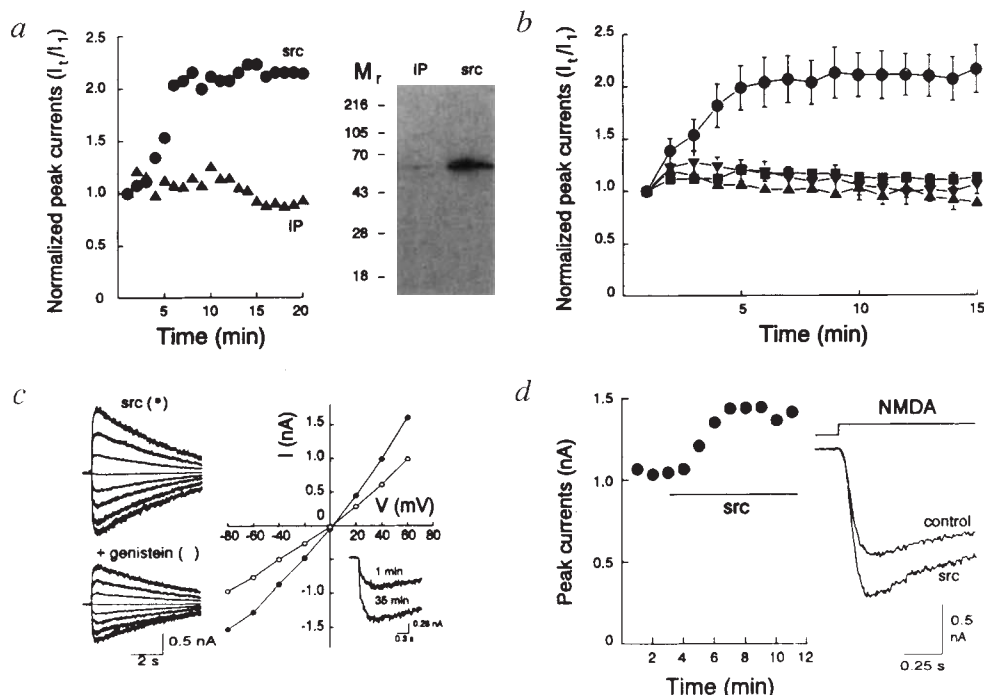


FIG. 2 The protein-tyrosine kinase pp60^{c-src} potentiates NMDA currents. Recordings were made using the whole-cell patch configuration: a, b and c, from spinal dorsal horn neurons; and d, from a hippocampal neuron. a, Normalized peak currents evoked by L-aspartate in two individual neurons are plotted in the graph. For one cell, the intracellular solution was supplemented with pp60^{c-src} (30 U ml⁻¹; src, ●). For the other cell, the pp60^{c-src} stock solution was immunoprecipitated (IP) with a specific anti-pp60^{c-src} antibody before being added to the intracellular solution (▲). Immunoprecipitation removed more than 98% of immunodetectable pp60^{c-src}; an immunoblot of samples taken before and after precipitation is shown on the right. The positions of molecular size standards (in K) are indicated. I_t/I_1 is the amplitude of the response at time t divided by that of the first response. b, Normalized NMDA currents recorded with intracellular solution containing pp60^{c-src} (30 U ml⁻¹; ●; $n=9$) or boiled pp60^{c-src} (30 U ml⁻¹; ▼; $n=5$) or immunoprecipitated solution (▲; $n=5$), or without kinase (■; $n=9$). c, Current-voltage (I - V) relationship for NMDA currents with pp60^{c-src} in the intracellular solution. Currents were evoked at membrane potentials from -80 to $+60$ mV. The I - V relationship was determined after the responses had stabilized (src, ●) and then in the presence of 100 μ M genistein (+genistein, ○). Records on the left are superimposed individual NMDA currents; I - V curves are plotted in the graph on the right. Traces in the inset on the lower right show the first current response (1 min) and a representative response at steady state (35 min). d, Peak currents evoked by rapid application²³ of NMDA (100 μ M) are plotted in the graph on the left. In this case pp60^{c-src} (30 U ml⁻¹) was actively perfused

through the recording electrode. The period of the perfusion is indicated by the horizontal bar in the graph. On the right representative currents recorded before ('control') and after ('src') intracellular perfusion with pp60^{c-src} are shown. NMDA was applied as indicated by the trace above the currents.

METHODS. The intracellular recording solution contained (in mM): CsCl (140), HEPES (10), BAPTA (10) and Mg-ATP (4), pH 7.25; osmolarity, 300–315 mosM. Series resistance was typically 5–8 M Ω . The first application of L-aspartate or NMDA was made within 1 min after starting whole-cell recording. Purified recombinant human pp60^{c-src} (Upstate Biotechnology, NY) was stored in a stock solution (3,000 U ml⁻¹) and added to the intracellular solution at a dilution of 1:100 just before use. The stock solution was immunoprecipitated for 2 h at 4 °C with agarose-coupled anti-pp60^{c-src} antibody (N-16, Santa Cruz Biotechnology). The solution was centrifuged and the supernatant used for the intracellular solution such that the final dilution of the stock was 1:100. Aliquots before and after immunoprecipitation were subject to SDS-PAGE (10% acrylamide). Proteins were transferred to a nitrocellulose membrane. Immunodetection with anti-src antibody, SRC2 (Santa Cruz Biotechnology), was achieved using enhanced chemiluminescence (ECL, Amersham). Boiled pp60^{c-src} was prepared by heating the stock solution in a boiling water bath for 30 min. Cultured hippocampal neurons were prepared from fetal mice²⁴. Active intracellular perfusion was accomplished by means of a second glass pipette (outer diameter, 0.75 mm) inserted into the recording electrode.

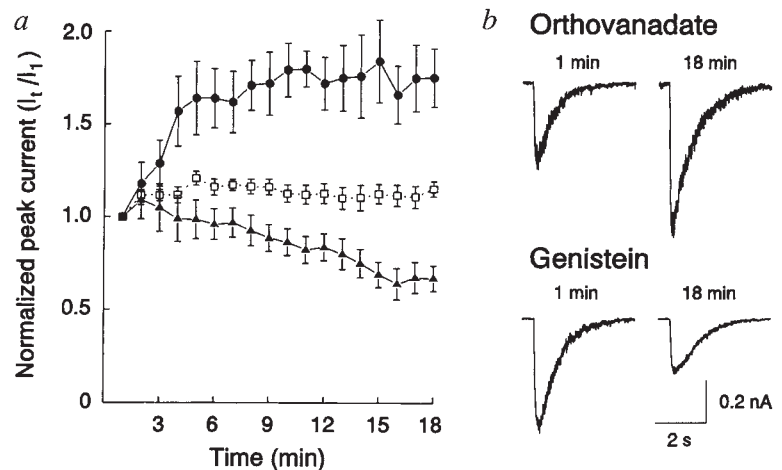


FIG. 3 Effects of intracellular application of the PTP inhibitor orthovanadate, or the PTK inhibitor genistein, on NMDA currents. **a**, I_t/I_1 is plotted for recordings made using standard whole-cell configuration from neurons recorded with intracellular solution containing sodium orthovanadate ($\bullet$; 100 μ M; $n=5$) or genistein ($\blacktriangle$; 100 μ M; $n=7$). For the control group ($\square$; $n=9$), the intracellular solution did not contain inhibitors. Data are means $\pm$ s.e.m. **b**, Representative current traces taken at the times indicated are shown from a neuron perfused with sodium orthovanadate (upper) and another perfused with genistein (lower).

as well as by PTKs was investigated by intracellular application of the PTP inhibitor sodium orthovanadate¹⁵ in cultured dorsal horn neurons. Orthovanadate (100 μ M) potentiated NMDA currents 1.5–2-fold (Fig. 3). The reversal potential of the currents was almost 0 mV and hence the conductance rather than the driving force appeared to be affected by orthovanadate. On the other hand, intracellular application of genistein (100 μ M; $n=7$ cells) caused NMDA currents to decline progressively to a stable level that was 60–70% of that of the control (Fig. 3).

Our results show that NMDA receptors can be modulated by protein-tyrosine kinases and protein-tyrosine phosphatases. This modulation could be due to phosphorylation/dephosphorylation of the NMDA receptors themselves or of regulatory protein(s) associated with the receptors. The increased whole-cell conductance produced by PTKs might result from recruiting previously inactive channels or enhancing the gating of active channels, possibly by increasing the frequency or duration of channel opening. We have observed that unitary conductance measured by fluctuation analysis of whole-cell currents is unaffected by PTKs (unpublished results).

Protein-tyrosine kinases and phosphatases are common elements in signalling pathways for many extracellular molecules such as growth factors¹⁶, cytokines¹⁷ and neurotransmitters^{18–20}. The modulation reported here of NMDA receptors by PTKs and PTPs may be important in physiological and pathophysiological processes in the central nervous system. $\square$

Received 17 September 1993; accepted 10 March 1994.

- Hunter, T. & Cooper, J. A. *Rev. Biochem.* **54**, 897–930 (1985).
- Fischer, E. H., Charbonneau, H. & Tonks, N. K. *Science* **253**, 401–406 (1991).
- Wager, K. R., Mei, L. & Huganir, R. L. *Curr. Opin. Neurobiol.* **1**, 65–73 (1991).
- Catarsi, S. & Drapeau, P. *Nature* **363**, 353–355 (1993).
- O'Dell, T. J., Kandel, E. R. & Grant, S. G. *Nature* **353**, 558–560 (1991).
- Grant, S. G. N. *et al. Science* **258**, 1903–1910 (1992).
- Walaas, S. I. & Greengard, P. *Pharmac. Rev.* **43**, 299–349 (1991).
- Raymond, L. A., Blackstone, C. D. & Huganir, R. L. *Trends Neurosci.* **16**, 147–153 (1993).
- Mayer, M. L. & Westbrook, G. L. *Prog. Neurobiol.* **28**, 197–276 (1987).
- Gasic, G. P. & Hollmann, M. E. *Rev. Physiol.* **54**, 507–536 (1992).
- Mayer, M. L. & Westbrook, G. L. *J. Physiol., Lond.* **354**, 29–53 (1984).
- Akiyama, T. *et al. J. Biol. Chem.* **262**, 5592–5595 (1987).
- Onoda, T. *et al. J. Nat. Prod.* **52**, 1252–1257 (1989).
- Brickell, P. M. *Crit. Rev. Oncogen.* **3**, 401–446 (1992).
- Swarup, G., Cohen, S. & Garbers, D. L. *Biochem. Biophys. Res. Commun.* **107**, 1104–1109 (1982).
- Schlessinger, J. & Ullrich, A. *Neuron* **9**, 383–391 (1992).
- Chiba, T. *et al. Nature* **362**, 646–648 (1993).
- Pan, M. G., Florio, T. & Stork, P. J. S. *Science* **256**, 1215–1217 (1992).
- Bading, H. & Greenberg, M. E. *Science* **253**, 912–914 (1991).
- Zachary, I., Gil, J., Lehmann, W., Sinnet-Smith, J. & Rozengurt, E. *Proc. natn. Acad. Sci. U.S.A.* **88**, 4577–4581 (1991).
- Horn, R. & Marty, A. *J. gen. Physiol.* **92**, 145–159 (1988).
- Salter, M. W. & Hicks, J. L. *J. Neurosci.* **14**, 1563–1575 (1994).
- Johnson, J. W. & Ascher, P. *Nature* **325**, 529–531 (1987).
- Wang, L. Y., Salter, M. W. & MacDonald, J. F. *Science* **253**, 1132–1135 (1991).

ACKNOWLEDGEMENTS. We thank H. Atwood, J. F. MacDonald and D. Rotin for critical comments on the manuscript, J. F. MacDonald and L. Y. Wang for experiments with hippocampal neurons, and J. L. Hicks for technical assistance. This work was supported by the MRC of Canada and the Nicole Fealdman Memorial Fund. M.W.S. is an MRC Scholar and Y.T.W. is an MRC Fellow.

Regulation of NMDA channel function by endogenous Ca^{2+} -dependent phosphatase

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PROTEIN kinases modulate the activity of several ligand-gated ion channels¹, including the NMDA (*N*-methyl-D-aspartate)² subtype of glutamate receptor. Although phosphorylation and dephosphorylation of glutamate receptors may participate in several lasting physiological and pathological alterations of neuronal excitability^{3–7}, the physiological control of this cycle for NMDA channels has not yet been established. Using cell-attached recordings in acutely dissociated adult rat dentate gyrus granule cells, we now demonstrate that inhibitors of an endogenous serine/threonine phosphatase prolong the duration of single NMDA channel openings, bursts, clusters and superclusters. Okadaic acid, a non-selective phosphatase inhibitor, prolongs channel openings only at a concentration that inhibits the Ca^{2+} /calmodulin-dependent phosphatase 2B (calcineurin)⁸, and is ineffective when Ca^{2+} entry through NMDA channels is prevented. Furthermore, FK506, an inhibitor of calcineurin^{9,10}, mimics the effects of okadaic acid. Thus in adult neurons, calcineurin, activated by calcium entry through native NMDA channels, shortens the duration of channel openings. Simulated synaptic currents¹¹ were enhanced after phosphatase inhibition, which is consistent with the importance of phosphorylation of the NMDA-receptor complex in the short- and long-term control of neuronal excitability.

Single-channel activity was measured in cell-attached patches from adult mammalian central nervous system neurons to investigate the functional regulation of NMDA channels by phosphorylation/dephosphorylation and to eliminate developmental components from our preparation. Furthermore, intracellular constituents, presumably lost during whole-cell 'rundown'¹², were maintained in such recordings, permitting endogenous regulation of NMDA channels.

Low concentrations of L-aspartate (100–250 nM) were used in the presence of saturating glycine (3 μ M) to ensure distinct

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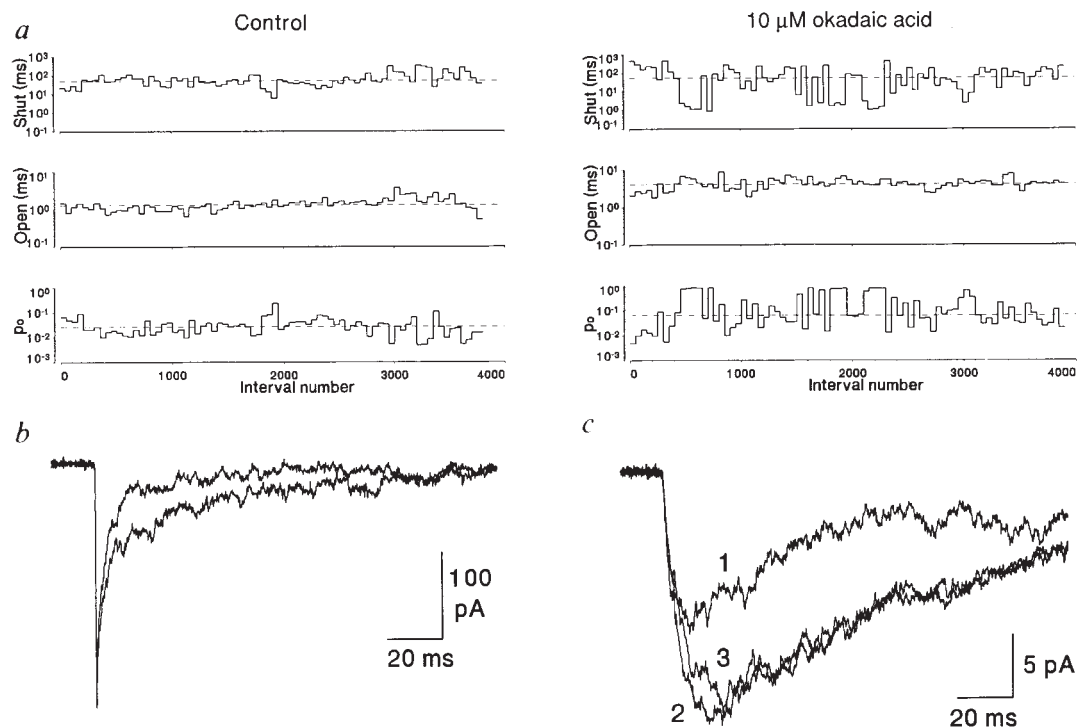
channel activations, to prevent pronounced desensitization, and to minimize non-NMDA channel activation^{4,13}. Assuming an NMDA reversal potential of ~ 0 mV, and a resting membrane potential of about -60 mV (ref. 4), the single NMDA channel conductance was ~ 50 pS for more than 90% of openings⁴. Within 2–5 min of bath application, the membrane-permeant phosphatase inhibitor okadaic acid¹⁴ ($10 \mu\text{M}$) dramatically altered NMDA channel openings in cell-attached recordings (Figs 1a, 2a and Table 1). All types of channel openings were prolonged, including the characteristic complex groupings into bursts, clusters and superclusters^{4,13}. In addition, okadaic acid increased the open probability (P_o) in 3 out of 7 experiments (Fig. 1a). In the remaining experiments, a few extremely long shut times interspersed with channel openings may have increased the variability of P_o (Table 1). Even in these latter cells, phosphatase inhibition caused larger swings towards both 'silent' and 'burst' periods, as indicated by the 135% increase in the coefficient of variation of the P_o in binned stability plots.

Prolonged burst and cluster lengths, even without a concomitant increase in the overall steady-state P_o , can lead to an augmented ensemble or synaptic current, as shown in Fig. 1b and c using summations of experimental records. Alignment of 135 individual successive superclusters¹¹ gave a summed ensemble current with an area enhanced 110% by okadaic acid (Fig. 1b). To circumvent complications arising from presynaptic effects of phosphatase inhibitors¹⁵, we have simulated postsynaptic NMDA currents using cell-attached single channel records. A

double exponential first latency distribution was used to approximate the kinetics of synaptic currents¹¹ (Fig. 1c). The area of the average simulated synaptic NMDA current was increased by 94% in okadaic acid even though the overall P_o was decreased by 29% in this neuron. Assuming the okadaic acid does not alter the Ca^{2+} permeability of the NMDA channel, this increase would nearly double the Ca^{2+} entry during each synaptic response. Under steady-state conditions, ambient levels of glutamate¹⁶ should also cause large increases in Ca^{2+} transients, as judged from the lengthening of total open times during bursts and clusters after phosphatase inhibition (Table 1).

A significant fraction of the inward current through NMDA channels is carried by calcium ions^{17,18}. Consequently, a local increase in cytoplasmic Ca^{2+} at the inner mouth of the channel could activate Ca^{2+} -dependent cellular events to produce inactivation (desensitization)¹⁹, possibly through interactions with the cytoskeleton²⁰. We examined the Ca^{2+} dependence of the okadaic acid effect on NMDA channel openings using cell-attached pipettes containing a nominally Ca^{2+} - and Mg^{2+} -free solution with 10 mM EDTA¹³. The bath solution contained normal (1.8 mM) Ca^{2+} in order to discriminate between effects of Ca^{2+} entry through NMDA channels and elevations of intraneuronal Ca^{2+} through other means such as influx through voltage-dependent Ca^{2+} channels. As expected¹³, removal of Ca^{2+} from the cell-attached pipette prolonged the mean open time by $20 \pm 7.8\%$ and increased the conductance by $50 \pm 12\%$ ($n=7$; compare raw traces in Fig. 2a and c). Okadaic acid, however, had no effect

FIG. 1 The effects of okadaic acid on dwell times and open probability of NMDA channels in cell-attached patches. a, Stability plots for shut times (top panels), open times (middle panels) and open probability (P_o) (lower panels). Each bin consisted of 50 consecutive events. The recordings are from a 5-min period in the control and a 6-min period in okadaic acid ($10 \mu\text{M}$). The phosphatase inhibitor shortened shut times, prolonged open times, and produced high P_o periods with a 135% increase in the overall P_o for this cell. Another cell with prolonged openings, but a 29% decrease in the overall P_o after okadaic acid treatment, was used for the simulations depicted in b and c. b, Of all superclusters observed during 4-min periods



before and after okadaic acid application, 135 consecutive individual superclusters were aligned and summed for each condition. Critical closed times for superclusters^{4,13} were 152 and 151 ms before and after exposure to okadaic acid, respectively. In simulated ensemble NMDA responses, prolonged channel openings by okadaic acid augmented the area of the current by 110%. c, A double exponential first latency distribution¹¹ ($\tau_1=13$ ms; relative amplitude, 63%; $\tau_2=176$ ms) was assigned to 200 subsets of 50 superclusters randomly selected from the same two sets of 135 superclusters used in b. Assuming that the latency to first openings is not affected (trace 2), phosphatase inhibition increased the total charge carried by the simulated synaptic current by 94% over control (trace 1). When the simulation was performed with a 29% longer latency to first openings to reflect the decrease in overall P_o (trace 3), the area of the current increased 90% over control.

METHODS. Procedures for the dissociation of neurons from adult (250–350 g) male Wistar rats have been described⁴. Fire-polished, Sylgard-coated, thick-walled borosilicate (Garner Glass) glass pipettes (10–25 M Ω) were filled with the recording/extracellular solution containing (in mM): Na_2SO_4 , 110; Cs_2SO_4 , 5; CaCl_2 , 1.8; HEPES, 10; D-glucose, 10; pyruvic acid, 1; tetrodotoxin (Calbiochem), 0.001 (final pH 7.25, osmolarity, 285–305 mOsm). In experiments in which Ba^{2+} replaced Ca^{2+} , sulphates were replaced with chloride salts. Cell-attached recordings were obtained at 22–25 $^\circ\text{C}$ using pipettes filled with 100–250 nM L-aspartic acid and 3 μM glycine dissolved in the extracellular solution. Stock solutions of okadaic acid (500 μM ; LC Services) were prepared in 100 μl dimethyl sulphoxide (final concentration $<0.08\%$ v/v). Details of the analysis using the PAT program (courtesy of J. Dempster) with a 50% threshold crossing algorithm for event detection are given elsewhere⁴.

TABLE 1 Okadaic acid effects on the mean dwell times of activated NMDA channels

	Control		10 μ M okadaic acid	
	Mean $\pm$ s.e.m.	<i>n</i>	Mean $\pm$ s.e.m.	<i>n</i>
Shut time (ms)	120.4 $\pm$ 47.5	7	149.8 $\pm$ 70.4	7
All openings (ms)	1.27 $\pm$ 0.23	7	2.04 $\pm$ 0.49*	7
Openings in bursts with 1 opening (ms)	1.07 $\pm$ 0.19	7	1.50 $\pm$ 0.36	7
Openings in bursts with ≥ 2 openings (ms)	1.41 $\pm$ 0.26	7	2.26 $\pm$ 0.54*	7
P_{open} overall ($\times 10^{-4}$)	33.9 $\pm$ 14.7	7	30.1 $\pm$ 8.0	7
Burst length (ms)	2.77 $\pm$ 0.52	7	6.91 $\pm$ 1.66*	7
Total open/burst (ms)	2.28 $\pm$ 0.44	7	4.65 $\pm$ 1.12*	7
Number of openings/burst	1.82 $\pm$ 0.13	7	2.36 $\pm$ 0.32*	7
P_{burst} ($\times 10^{-3}$)	53.0 $\pm$ 21.5	7	231.4 $\pm$ 114.5	7
Burst P_{open}	0.87 $\pm$ 0.02	7	0.85 $\pm$ 0.04	7
Cluster length (ms)	26.43 $\pm$ 9.79	7	47.01 $\pm$ 13.26*	7
Total open/cluster (ms)	4.75 $\pm$ 1.07	7	10.76 $\pm$ 2.23*	7
Number of openings/cluster	3.63 $\pm$ 0.49	7	6.26 $\pm$ 0.78*	7
P_{cluster} ($\times 10^{-3}$)	103.3 $\pm$ 40.4	7	234.5 $\pm$ 107.7	7
Cluster P_{open}	0.65 $\pm$ 0.02	7	0.62 $\pm$ 0.05	7
Supercluster length (ms)	126.9 $\pm$ 20.17	5	332.5 $\pm$ 66.5*	6
Total open/supercluster (ms)	12.9 $\pm$ 2.4	5	29.7 $\pm$ 4.4*	6
Number of openings/supercluster	11.0 $\pm$ 2.07	5	18.6 $\pm$ 3.7*	6
$P_{\text{supercluster}}$ ($\times 10^{-3}$)	235.8 $\pm$ 74.2	5	171.7 $\pm$ 45.0	6
Supercluster P_{open}	0.40 $\pm$ 0.05	5	0.45 $\pm$ 0.06	6

NMDA channels were activated by 100–250 nM L-aspartate in cell-attached recordings.

* Values show a significant difference ($P < 0.05$, two-tailed *t*-test with correction for unequal variances) before and after perfusion of the phosphatase inhibitor. Details of the analysis have been described⁴.

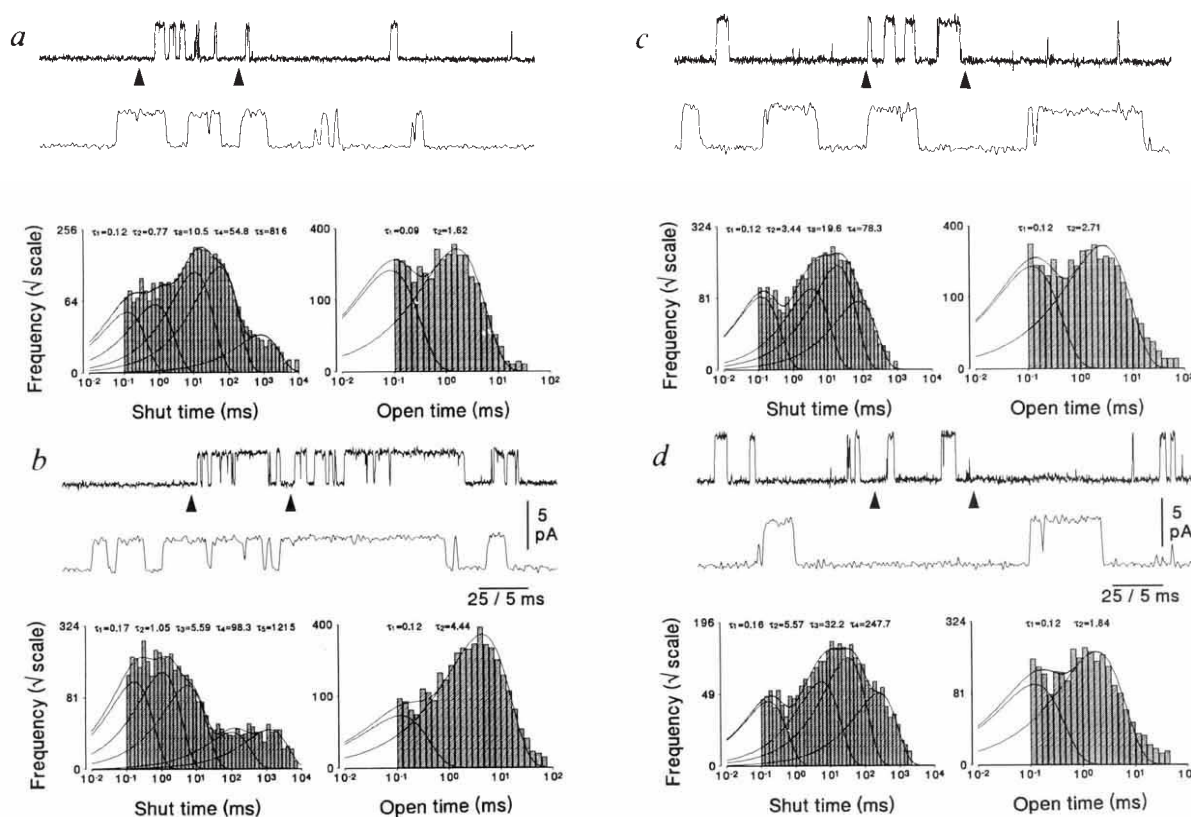


FIG. 2 Representative raw traces and dwell time histograms of cell-attached single NMDA channel currents from two patches, each obtained before (a, c) or after (b, d) application of 10 μ M okadaic acid in the presence (a, b) or absence (c, d) of extracellular calcium. The patches were held at identical holding potentials (-60 mV). In the upper portion of each panel the arrowheads indicate the segment of the sweep that is portrayed on an expanded timescale below. a, b, In the presence of 10 μ M okadaic acid and 1.8 mM $[\text{Ca}^{2+}]_o$, mean single channel open time, shut time and burst duration increased from 1.39 to 4.30 ms, from 69.6 to 90.1 ms, and from 1.55 to 9.45 ms, respectively, while the mean single channel current remained relatively constant (from 3.47 ± 0.11 pA to 3.25 ± 0.16 pA). The control shut time distribution (a) is fitted with the sum of five exponential components drawn separately and as a sum (time constants are indicated). An increased percentage

of short shut times can be observed in the five exponential components after treatment of the cell with okadaic acid (b). The distribution of all apparent openings longer than 100 μ s was fitted with the sum of two exponentials. c, d, When the patch electrode contained a Ca^{2+} -free medium and 10 mM EDTA¹⁰, the mean open time, shut time and burst duration changed in response to okadaic acid (10 μ M) from 2.34 to 1.68 ms, from 24.4 to 65.6 ms, and from 4.99 to 5.03 ms, respectively, while the single channel currents again remained relatively constant (4.38 ± 0.17 pA to 4.43 ± 0.11 pA). Note that under these experimental conditions shut time distributions were best fitted with the sum of four, as opposed to five, exponential components for both control (c) and okadaic acid treatment (d), whereas the distribution of all apparent openings longer than 100 μ s was fitted with the sum of two exponentials with the indicated time constants.

on NMDA channel openings when Ca^{2+} was prevented from permeating the channel (Figs 2c, d and 3a–d).

We have considered the possibility that okadaic acid may have altered the Mg^{2+} block²¹ of the channel, as trace amounts of Mg^{2+} ($\sim 4 \mu\text{M}$ ¹³) may have been present in our ' Mg^{2+} -free' solutions. When $500 \mu\text{M}$ EDTA was added to both the Ca^{2+} -containing bath and pipette solutions to chelate any residual Mg^{2+} , okadaic acid ($10 \mu\text{M}$) was still effective in prolonging NMDA channel openings ($n=4$). Thus, either there was no residual Mg^{2+} block, or alterations in the Mg^{2+} sensitivity of the channel play no part in the okadaic acid effect.

To clarify further the relationship between Ca^{2+} permeation and phosphatase activation, both bath and pipette Ca^{2+} were replaced by equimolar Ba^{2+} which also permeates the NMDA channel¹⁷ but does not activate²² the Ca^{2+} /calmodulin-dependent phosphatase 2B. In the presence of Ba^{2+} , the single channel conductance and channel openings, including superclusters, were similar to those recorded in extracellular Ca^{2+} , but $10 \mu\text{M}$ okadaic acid did not prolong channel openings ($n=4$; Fig. 3a–d).

A variety of intracellular phosphatases are inhibited by okadaic acid to varying degrees. Antagonism of several cytosolic

phosphatases, including phosphatase types 1, 2A and 2B (refs 8, 14) may have led to the augmentation of NMDA channel activity by $10 \mu\text{M}$ okadaic acid in the presence of extracellular Ca^{2+} . Of these three phosphatases, phosphatase 2B is the least inhibited ($\text{IC}_{50} 5 \mu\text{M}$; ref. 8) whereas phosphatase 2C, alkaline phosphatase, a protein tyrosine phosphatase, and eight different protein kinases (including protein kinase C) are unaffected^{14,22}. By doing the experiments in the presence of 1.8 mM Ca^{2+} but with low concentrations of okadaic acid (0.5 – $1.0 \mu\text{M}$), conditions that should inhibit only phosphatases 1 and 2A, but not calcineurin^{8,14}, we found no significant effect of okadaic acid on channel openings ($n=7$; Fig. 3a–d). Consistent with our findings, it has been shown^{5,6} that NMDA receptor-mediated synaptic events recorded in hippocampal slices are unaffected by calyculin A, an inhibitor of phosphatases 1 and 2A but not of phosphatase 2B.

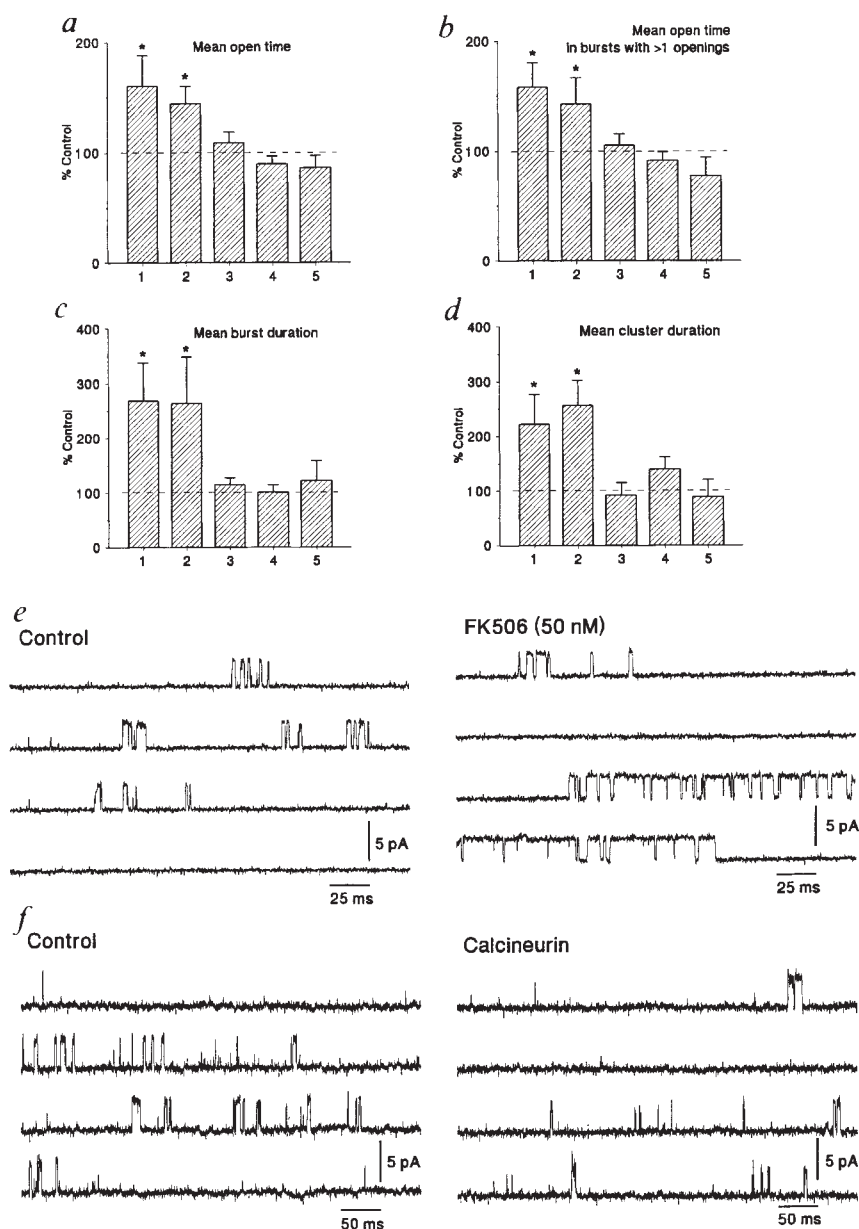
To confirm that calcineurin was the phosphatase inhibited in the previous experiments, we examined the effects of FK506, a potent and selective calcineurin inhibitor^{9,10}, on cell-attached patches, and that of calcineurin itself on inside-out patch recordings. The complex of the immunosuppressant drug FK506

FIG. 3 a–d, Phosphatase inhibitors prolong NMDA channel openings. In each trial, paired experimental:control ratios were calculated and were expressed as per cent of control. Averaged ratios ($\pm$ s.e.m.) within each group are plotted, and significant differences ($P < 0.05$; Kruskal–Wallis test) are indicated by asterisks. Increases in mean open time (a), mean open time in bursts with more than one opening (b), mean burst duration (c) and mean cluster duration (d) are shown. The experimental conditions corresponding to the abscissae in a–d are:

Bar	Inhibitor	$[\text{Ca}^{2+}]_0$	$[\text{Ba}^{2+}]_0$	n
1	Okadaic acid, $10 \mu\text{M}$	1.8 mM	0 mM	7
2	FK506, 10 – 50 nM	1.8 mM	0 mM	7
3	Okadaic acid, 0.5 – $1.0 \mu\text{M}$	1.8 mM	0 mM	7
4	Okadaic acid, $10 \mu\text{M}$	0 mM	1.8 mM	7
5	Okadaic acid, $10 \mu\text{M}$	0 mM	1.8 mM	4

e–f, Recordings illustrate changes in NMDA channel openings produced by a specific calcineurin inhibitor (e) and by calcineurin itself (f). FK506 was dissolved in dimethyl sulphoxide and diluted to a final concentration of 10 – 50 nM . Five minutes after FK506 perfusion, the mean open time in this cell-attached recording increased from 1.99 to 3.44 ms while P_0 increased from 0.006 to 0.010 . The mean burst, cluster and supercluster durations were prolonged by 227 , 122 and 126% , respectively. Application of $40 \mu\text{g ml}^{-1}$ calcineurin, $20 \mu\text{M}$ calmodulin and $40 \mu\text{M}$ Ca^{2+} to the cytoplasmic side of an inside-out patch (f) decreased the mean open time by 51% and P_0 by 89% . The burst duration was reduced by 44% , the cluster duration by 50% and the supercluster length by 36% . Note different timescales in e and f.

METHODS. For calcineurin experiments, inside-out patches were excised in normal extracellular solution (no added Ca^{2+}). Fire-polished and Sylgard coated borosilicate glass pipettes (4 – $6 \text{ M}\Omega$) were filled with the extracellular solution, including 1.8 mM CaCl_2 , 100 nM L-aspartic acid and $3 \mu\text{M}$ glycine. The patches were moved into a well containing (in mM): caesium gluconate 140 , HEPES 10 , Mg-ATP 4 and CaCl_2 0.001 , and were continually perfused with this solution (at -60 mV) to prevent channel rundown. Patches showing substantial rundown were discarded. The NMDA channels were identified by their conductance and their sensitivity to $10 \mu\text{M}$ ketamine, which blocks NMDA channels by a hydrophobic pathway when applied to the inside surface of the patch (J. F. MacDonald, personal communication).



with its immunophilin-binding protein FKBP has been reported to inhibit calcineurin selectively in many tissues including brain²³. Like high concentrations of okadaic acid, application of 10–50 nM FK506 to cell-attached patches prolonged the simple and complex openings of NMDA channels ($n=7$; Fig. 3a–e). The prolongations of burst, cluster and supercluster durations and the increases in total open time during these openings observed with FK506 were comparable to those seen in the presence of 10 μ M okadaic acid. As with okadaic acid, FK506 had no effect on the overall P_0 averaged across 7 cells, whereas P_0 was increased in 4 out of 7 experiments. In the remaining cells, the swings between high and low P_0 periods were enhanced by inhibition of calcineurin as reflected in the FK506-induced, 147% increase in the coefficient of variation in stability plots of P_0 . In contrast, exposure of the cytoplasmic surface of inside-out patches to 40 μ g ml⁻¹ calcineurin and half-saturated calmodulin (20 μ M calmodulin and 40 μ M Ca²⁺) shortened channel openings ($n=3$; Fig. 3f). After calcineurin was added, the mean open time, burst duration, cluster duration and overall P_0 decreased by 28, 54, 40 and 65%, respectively.

In summary, a specific serine/threonine phosphoprotein phosphatase appears to modulate the openings of native NMDA channels. Calcium ions entering through the channel activate phosphatase 2B (calcineurin), which in turn acts either directly or indirectly on the channel complex to diminish its activity. Calcineurin induction secondary to NMDA receptor activation²⁴ may alter the state of microtubule and/or actin polymerization²⁵ and thereby affect channel activity²⁰. As calcineurin is absent in mammalian neurons until postnatal day 4 (ref. 26), other protein phosphatases may be involved during early developmental stages of intracellular machinery and receptor subunit assembly²⁷. In adult neurons, Ca²⁺-dependent phosphatase(s) may ensure that Ca²⁺ entry through NMDA channels adequately initiates Ca²⁺-dependent second messenger actions³ while preventing pronounced intracellular Ca²⁺ build-up, reducing the threat of excitotoxic cell damage. Loss of calcineurin subunits, as occurs following ischaemia²⁸, could mean a loss of negative feedback control over large or sustained increases in intracellular Ca²⁺ and may underlie the anoxia-induced selective potentiation of NMDA responses²⁹, contributing to post-ischaemic cell damage. The degree of NMDA receptor activation by synaptically released or ambient levels of glutamate critically depends on channel kinetics^{16,30}, making the phosphorylation/dephosphorylation-dependent control of channel openings a major component in regulating short- and long-term changes of neuronal excitability. □

Received 26 August 1993; accepted 5 May 1994.

- Raymond, L. A., Blackstone, C. D. & Hagan, R. L. *Trends Neurosci.* **16**, 147–153 (1993).
- Chen, L. & Huang, L.-Y. M. *Nature* **356**, 521–523 (1992).
- Bliss, T. V. P. & Collingridge, G. L. *Nature* **361**, 31–39 (1993).
- Köhr, G., De Koninck, Y. & Mody, I. J. *Neurosci.* **13**, 3612–3627 (1993).
- Figurov, A., Boddeke, H. & Müller, D. *Eur. J. Neurosci.* **5**, 1035–1041 (1993).
- Mulkey, R. M., Herron, C. E. & Malenka, R. C. *Science* **261**, 1051–1055 (1993).
- Tingley, W. G., Roche, K. W., Thompson, A. K. & Hagan, R. L. *Nature* **364**, 70–73 (1993).
- Bialojan, C. & Takai, A. J. *Biochem.* **256**, 283–290 (1988).
- Liu, J. et al. *Cell* **66**, 807–815 (1991).
- Clipstone, N. A. & Crabtree, G. R. *Nature* **357**, 695–697 (1992).
- Edmonds, B. & Colquhoun, D. *Proc. R. Soc.* **250**, 279–286 (1992).
- MacDonald, J. F., Mody, I. & Salter, M. W. J. *Physiol., Lond.* **414**, 17–34 (1989).
- Gibb, A. J. & Colquhoun, D. J. *Physiol., Lond.* **456**, 143–179 (1992).
- Haystead, T. A. J. et al. *Nature* **337**, 78–81 (1989).
- Abdul-Ghani, M., Kravitz, E. A., Meiri, H. & Rahamimoff, R. *Proc. natn. Acad. Sci. U.S.A.* **88**, 1803–1807 (1991).
- Sah, P., Hestrin, S. & Nicoll, R. A. *Science* **246**, 815–818 (1989).
- Mayer, M. L. & Westbrook, G. L. J. *Physiol., Lond.* **394**, 501–527 (1987).
- Schneggenburger, R., Zhou, Z., Konnerth, A. & Neher, E. *Neuron* **11**, 133–143 (1993).
- Legendre, P., Rosenmund, C. & Westbrook, G. L. J. *Neurosci.* **13**, 674–684 (1993).
- Rosenmund, C. & Westbrook, G. L. *Neuron* **10**, 805–814 (1993).
- Nowak, L., Bregestovski, P., Ascher, P., Herbert, A. & Prochiantz, A. *Nature* **307**, 462–465 (1984).
- Klee, C. B., Draetta, G. F. & Hubbard, M. J. *Adv. Enzym.* **61**, 149–200 (1988).
- Steiner, J. P. et al. *Nature* **358**, 584–587 (1992).
- Halpain, S. & Greengard, P. *Neuron* **5**, 237–246 (1990).
- Goto, S. et al. *J. Neurochem.* **45**, 276–283 (1985).
- Polli, J. W., Billingsley, M. L. & Kincaid, R. L. *Dev. Brain Res.* **63**, 105–119 (1991).
- Watanabe, M., Inoue, Y., Sakimura, K. & Mishina, M. *Neuroreport* **3**, 1138–1140 (1992).

- Morioka, M. et al. *J. Neurochem.* **58**, 1798–1809 (1992).
- Crepel, V., Hammond, C., Krnjevic, K., Chinea, P. & Ben-Ari, Y. *J. Neurophys.* **69**, 1774–1778 (1993).
- Lester, R. A. J. & Jahr, C. E. *J. Neurosci.* **12**, 635–643 (1992).

ACKNOWLEDGEMENTS. We thank Y. De Koninck for help with synaptic simulations and for discussion, and G. R. Crabtree for FK506. This research was supported by a Howard Hughes Predoctoral Fellowship to D.N.L. and NINDS grants to I.M.

Cyclosporin A inhibits growth of autocrine tumour cell lines by destabilizing interleukin-3 mRNA

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IN T cells, cyclosporin A (CsA) exerts its immunosuppressive effect by preventing transcriptional induction of the expression of interleukin(IL)-2. This is achieved by a mechanism that involves binding of a CsA-cyclophilin complex to calcineurin, which in turn inhibits the phosphatase-controlled translocation of transcription factor NFAT to the nucleus^{1–8}. We have previously identified IL-3 as an autocrine oncogenic regulator in tumour cell lines generated by introducing the v-H-ras oncogene into IL-3-dependent mast cells^{9–12}. Here we report that CsA specifically blocks autocrine tumour cell growth. The mechanism involves down-regulation of IL-3 expression by destabilization of the messenger RNA and requires ongoing transcription. Transcripts from exogenous IL-3 genes lacking the (A + U)-rich element (ARE) in the 3' untranslated terminal repeat could not be destabilized, suggesting that at least part of this sequence, which is known to mediate decay of short-lived mRNA¹³, participates in a CsA-sensitive regulatory mechanism.

Tumour cell lines derived from v-H-ras-expressing PB-3c mast cells secrete IL-3 which they use in an autocrine fashion. Growth of those lines is sensitive to anti-IL-3 antibody⁹. To test for possible inhibition of growth by CsA, V2D1 tumour cells were plated in methyl cellulose in the presence of CsA or antibody. The data in Fig. 1a show that CsA was as inhibitory as anti-IL-3, and the drug effect could be reversed by exogenous IL-3 (Fig. 1b). The same pattern was observed with several other PB-3c-derived tumour cell lines, including 15V4T2 and 15V4T3 (data not shown). CsA had no effect on normal, parental PB-3c cells requiring exogenous IL-3 for growth (Fig. 1c). A v-abl-transformed PB-3c clone (8abl) was also tested. These cells grow independently of IL-3 through a non-autocrine mechanism¹¹. They were insensitive to both drug and antibody inhibition (Fig. 1d). Taken together, the results from Fig. 1 indicate that CsA may act by blocking the autocrine IL-3 loop.

We next studied IL-3 mRNA levels in V2D1 cells following overnight treatment with CsA and observed a dose-dependent down-regulation (Fig. 2a). This was confirmed with tumour cell lines 15V4T2 and 15V4T3 (Fig. 2b). IL-4 and IL-6 transcripts, also expressed in V2D1 cells, were not affected by CsA (Fig. 2c), reflecting specificity of the drug effect. The immunosuppressant FK506, which inhibits calcineurin in a way similar to CsA but through a different binding protein⁶, was also inhibitory, suggesting that both drugs may act, as in T cells, through calcineurin (Fig. 2d).

In induced T cells, CsA inhibits IL-2 expression by preventing transcription¹. But nuclear run-on experiments showed that CsA did not inhibit IL-3 transcription in V2D1 or 15V4T2 cells (Fig. 3a). The same holds true for FK506 (data not shown). For

comparison, we also analysed IL-3 and IL-2 expression in the 9/6 murine T-cell line¹⁴ stimulated by ionomycin and the phorbol ester TPA (12-*O*-tetradecanoylphorbol-13-acetate) which results in transcriptional induction of IL-2. Here, CsA inhibited expression of both lymphokines at the transcriptional level (Fig. 3b). We concluded that down-regulation of IL-3 in the mast cell tumour lines cannot be explained by inhibition of transcription.

We have previously shown that IL-3 production in V2D1 cells is the result of a post-transcriptional induction mechanism. This was shown by nuclear run-on experiments, in which V2D1 cells were compared with their precursor cells¹². This is confirmed here (Fig. 3c, left panel) in tumour line 15V4T2, where the rate of nuclear transcription is very similar to that of the non-IL-3-producing precursor 15V4 (ref. 11). We found further evidence for a post-transcriptional mechanism from investigations of the stability of mRNA in the presence of actinomycin D, when IL-3 transcripts remained stable over the 5-h period tested (Fig. 3c, right panel). This is in contrast to normal PB-3c cells, in which induced IL-3 mRNA has a half-life of less than 30 min¹⁵. Figure 3c, right panel, shows that IL-4 mRNA is also stable in the tumour, but only IL-3 mRNA was destabilized by CsA.

We therefore analysed the effect of CsA on mRNA stability by a transgene approach. We introduced a genomic IL-3 construct into V2D1 cells, which contains an 8.6-kilobase (kb) *EcoRI* fragment including 5.5 kb of 5' flanking sequences¹⁶. This construct contains all the 5' and 3' regulatory sequences necessary for ionomycin induction and stabilization (data not shown), but lacks the CsA-sensitive enhancer element situated between the IL-3 and GM-CSF genes¹⁷. To distinguish the exogenous

from the endogenous transcripts by RNase protection assay, the transfected gene was marked by two silent point mutations in the third exon (Fig. 4a). Additionally, the construct carries *hph*, a gene selectable by hygromycin B that can also be used as internal control for measuring gene expression. The resulting hygromycin-B-resistant line is termed V2D1-M1. Figure 4b (lanes 6, 12, 18) shows the IL-3 expression pattern from untreated V2D1-M1 cells with the expected three protected fragments. The upper band corresponds to the endogenous transcripts, and the two bands below to the transcripts from the transfected gene.

When V2D1-M1 cells were treated with actinomycin D and the stability of IL-3 mRNA was monitored for five hours, it

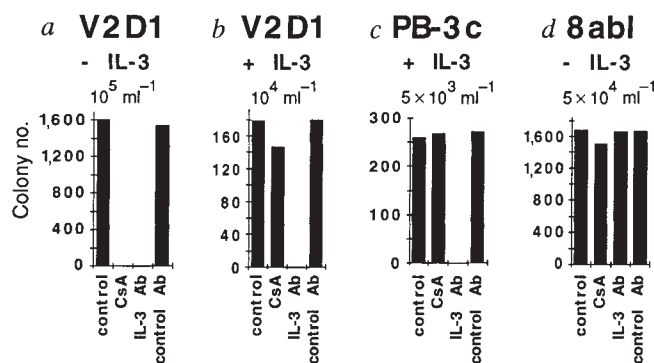


FIG. 1. CsA inhibits colony formation of autocrine mast cell tumour lines in methyl cellulose. V2D1 cells were plated in methyl cellulose in the absence (a) or presence (b) of IL-3. The effects of CsA and a monoclonal anti-IL-3 antibody¹⁹ were tested under both conditions. Also tested was colony formation of IL-3-dependent parental PB-3c cells (c), and a v-abl-transformed, IL-3-independent PB-3c subclone, 8abl (d)¹¹. METHODS. PB-3c is an IL-3-dependent, non-tumorigenic mastocyte line originating from mouse bone marrow²⁰. V2D1 is an autocrine tumour cell line derived from PB-3c cells by infecting with a defective retrovirus expressing v-H-ras^{9,11}. Methyl cellulose cloning was done in 1-ml cultures as described⁹. Autocrine PB-3c-derived tumour cell lines, like other autocrine cells, are characterized by a nonlinear relationship between cloning efficiency and the number of cells plated in methyl cellulose in the absence of the growth factor; no colonies are formed below a critical cell concentration unless IL-3 is added. Thus, the appropriate number of cells required for colony formation in the absence of IL-3 was first determined for each tumour (data not shown). Monoclonal anti-IL-3 antibody-secreting¹⁹ hybridoma cells (HB 10652) were obtained from American Type Culture Collection and 1% of the supernatant of confluent cells was used. Normal mouse serum was used as control. The 1-ml cultures contained, where indicated, 500 ng CsA (Sandoz Pharma) or 0.8% of conditioned medium from confluent X63-IL3 cells²¹ as the source of IL-3. Colony formation was scored after 10–15 days. Cell numbers plated are indicated above each graph.

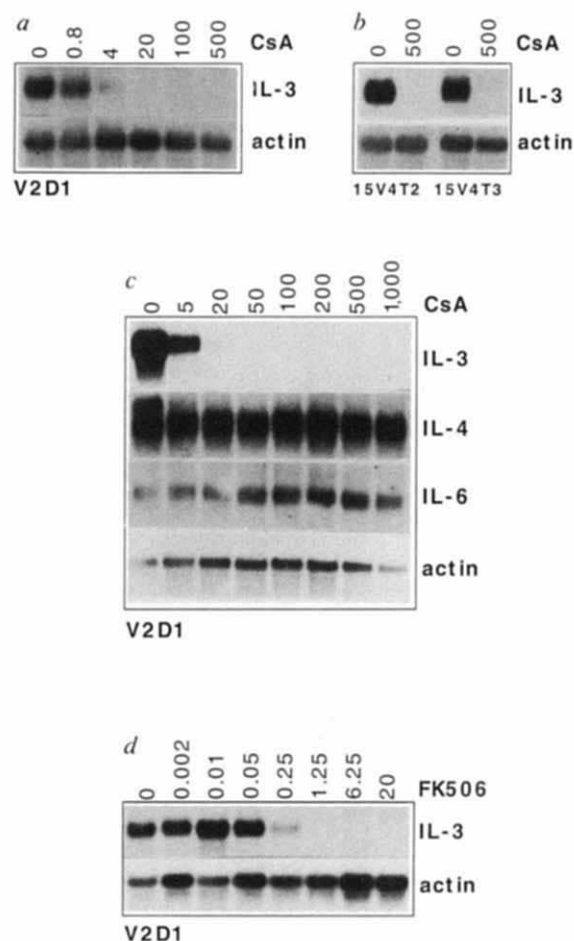


FIG. 2. Northern blot analysis from CsA- and FK506-treated tumour cell lines. The number on top of each lane indicates the drug concentration in the medium (ng ml⁻¹). An actin probe was used for rehybridization to control the amount of RNA loaded. a and b, Inhibitory effect of CsA on IL-3 expression in the cell lines indicated. c, Lack of CsA effect on IL-4 and IL-6 expression in V2D1 cells. d, Inhibitory effect of FK506 on IL-3 mRNA expression in V2D1 cells.

METHODS. 10⁵ cells per ml in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS and IL-3 were treated overnight with the indicated drug concentrations. FK506 was obtained from Fujisawa Ltd. Northern blot: cytoplasmic RNA was isolated as described in ref. 22; poly (A)⁺ mRNA was prepared using oligo(dT) cellulose, and hybridizations were done as described²¹. The probes used were: pSP65-multi-CSF containing a 368-bp *HindIII/XbaI* IL-3 cDNA fragment²³; pSPT18-IL-4 containing the 373-bp *RsaI* IL-4 cDNA fragment²⁴; a random-primed probe for IL-6 generated from the entire IL-6 cDNA subcloned into the pUC as an *EcoRI* fragment²⁵; pGEM-actin containing a 567-bp *PstI* fragment of chicken β -actin cDNA²¹. Before rehybridization, filters were washed in 70% formamide for 30 min at 65 °C followed by rinsing in 2 × SSC buffer.

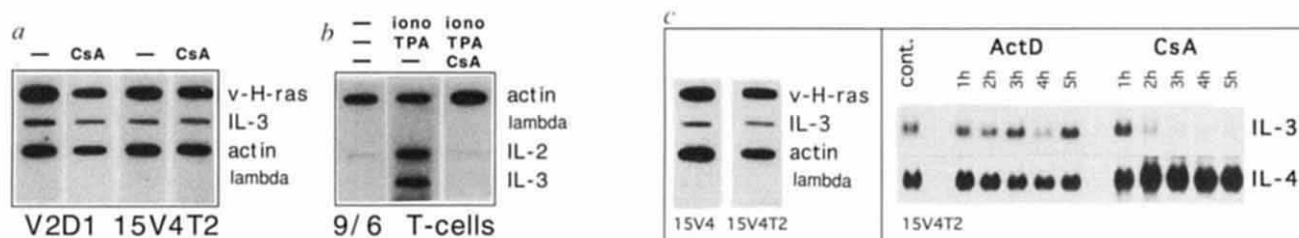


FIG. 3 Effect of CsA on nuclear and cytoplasmic IL-3 mRNA. **a**, Effect of CsA on the nuclear transcripts in V2D1 and 15V4T2; and **b**, the effect on 9/6 T-helper cells¹⁴. V2D1 and 15V4T2 cells were treated with 500 ng ml⁻¹ CsA in the presence of IL-3. 9/6 cells were stimulated with 2 μ M ionomycin (iono) and 20 ng ml⁻¹ TPA for 6.5 h in the presence of IL-2. CsA (500 ng ml⁻¹) was added 15 min before the stimulation. **c**, left panel, Nuclear run-on analysis of 15V4, the precursor, and 15V4T2, the tumour derived from it; right panel, Northern blot analysis of poly(A)⁺ RNA from 15V4T2.

METHODS. Nuclear run-on: nuclear preparation, transcription and isolation of labelled RNA was carried out as described¹¹. Aliquots of 3 μ g each of the following cDNA probes were denatured and slot-blotted onto

reinforced nitrocellulose filters (Schleicher & Schuell): control λ -phage DNA (Boehringer); pGEM-v-H-ras, carrying the 720-bp *Bam*HI fragment of the v-H-ras gene⁹; the IL-2 plasmid used was pSP64-IL-2, carrying the 337-bp *Hind*III/*Pst*I fragment of IL-2 cDNA²⁶. β -Actin and IL-3 probes were the same as those used for the northern blot. Filters were washed with 2 $\times$ SSC/0.1% SDS, 0.2 $\times$ SSC/0.1% SDS, and 0.1 $\times$ SSC/0.1% SDS at 65 $^{\circ}$ C for 30 min each, then rinsed twice with 2 $\times$ SSC and treated with 20 μ g ml⁻¹ RNase A in 2 $\times$ SSC for 15 min at 26 $^{\circ}$ C. The treatment was repeated, filters were rinsed twice with 2 $\times$ SSC and autoradiographed. Northern blot analysis was carried out as described for Fig. 2.

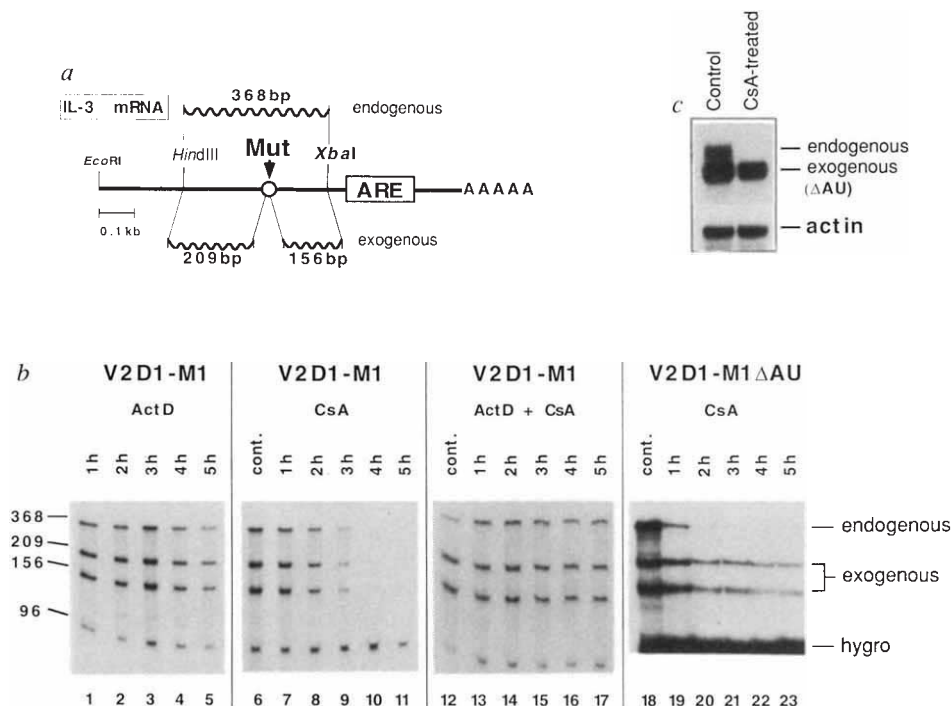
became obvious that the endogenous transcript in V2D1, as in the tumour 15V4T2 (Fig. 3c), is remarkably stable (Fig. 4b, lanes 1–5). It is noteworthy that the mRNA transcribed from the transfected gene has a half-life comparable to the endogenous mRNA and is apparently co-regulated. When these cells were treated with CsA, the transcripts from both the exogenous and endogenous genes disappeared with similar kinetics (Fig. 4b, lanes 6–11). In conjunction with the results from the nuclear run-on experiments, we conclude that the intrinsically stable IL-3 transcripts become destabilized by CsA. Co-treatment of cells with CsA and actinomycin D prevented mRNA destabilization (Fig. 4b, lanes 12–17). The requirement for continued transcription suggests a role for a short-lived macromolecule and argues that the CsA effect is indirect.

As AREs are major regulators of short-lived mRNAs¹³, we examined the role of these sequences in CsA sensitivity. An IL-3 gene lacking the ARE was constructed and introduced into V2D1 cells, generating V2D1-M1 Δ AU. The deletion removed a 216-base-pair (bp) *Nco*I/*Sty*I fragment containing the ARE with its AUUUA motifs and an additional 68 nucleotides¹⁶ from the 3' UTR. Cells were examined for responsiveness to CsA by RNase protection assay (Fig. 4b, lanes 18–23) and by northern blot analysis (Fig. 4c). Unlike full-length transcripts from the endogenous gene analysed in the same cells, mRNA expressed from the ARE-deletion construct could not be destabilized by CsA. This conclusion was confirmed by two independent transfection experiments. These results suggested that sequences linked to the ARE are required in *cis* for the CsA effect. But it

FIG. 4 Effect of CsA on IL-3 transgenes.

a, Schematic representation of IL-3 mRNA¹⁶. 'Mut' (circle) indicates the two adjacent nucleotides, which have been altered from T to G (position 1,894) and C to A (position 1,897), located in exon 3 of the IL-3 gene¹⁶. Also indicated is the 3' ARE. The 368-bp *Hind*III/*Xba*I fragment (Fig. 2 legend) was used as a riboprobe in the RNase protection assay. From the endogenous transcript, this probe protects a 368-bp fragment. Because of the mutation in exon 3 in both the full-length (pIL3-M1) and the ARE-deleted (pIL3-M1 Δ AU) constructs, two fragments of 209 bp and 156 bp are obtained after RNase treatment, representing exogenous IL-3 mRNA. **b**, Effect of actinomycin D (5 μ g ml⁻¹) alone (lanes 1–5), CsA alone (lanes 6–11), and both together (lanes 12–17) on V2D1-M1 cells. Lanes 18–23 show the effect of CsA on V2D1-M1 Δ AU. **c**, Northern blot analysis of V2D1-M1 Δ AU cells. The control shows two IL-3 mRNA species, where the upper band represents the endogenous RNA and the lower band the transgene, which is 216 bp shorter. 'CsA-treated' lane shows the IL-3 mRNA, from the shorter transgene.

METHODS. pIL3-M1 and pIL3-M1 Δ AU were introduced into V2D1 cells by electroporation, selected for resistance against hygromycin B (1 mg ml⁻¹), generating the lines V2D1-M1 and V2D1-M1 Δ AU, respectively. Electroporation was at 300 V and 960 μ F capacitance²⁷. Solution hybridization and RNase protection assays have been described²⁷. For



the RNA-loading control (hygro), labelled RNA transcribed from pGEMhygro carrying a 96-bp fragment from *hph* cDNA²⁸ was used. This gives rise to a protected fragment of 96 bp in both cell lines carrying the *hph* gene as the selectable marker. CsA treatment, RNA isolation and hybridizations in **c** were as for Fig. 2.

may well be that other sequence elements, located upstream to the ARE or even in the 5' UTR, may also play a role. A detailed mutational analysis of the ARE including its characteristic AUUUA motifs and outside sequences will clarify this issue.

In conclusion, we report a novel inhibitory effect of the immunosuppressive drug CsA acting at the post-transcriptional level by a process depending on an intact ARE. We have previously shown that IL-3 mRNA stabilization occurs during allergic IL-3 activation of normal mast cells^{15,18}. A post-

transcriptionally regulated IL-3 production mechanism also operates in the generation of the PB-3c-derived tumours analysed here, as shown by nuclear run-on experiments and mRNA stability data (ref. 12 and Fig. 3c). As both CsA and FK506 are active, the effect could be transmitted through calcineurin, the common mediator of these drugs in T cells⁶. As pharmacological destabilization of transcripts coding for auto-crine or other oncogenic regulators is potentially important therapeutically, it will be worth investigating whether mRNAs from other genes respond similarly. □

Received 13 January; accepted 31 March 1994.

- Krönke, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5214–5218 (1984).
- Flanagan, W. M., Corthésy, B., Bram, R. J. & Crabtree, G. R. *Nature* **352**, 803–807 (1991).
- Sigal, N. H. & Dumont, F. J. A. *Rev. Immun.* **10**, 519–537 (1992).
- O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J. & O'Neill, E. A. *Nature* **357**, 692–694 (1992).
- Clipstone, N. A. & Crabtree, G. R. *Nature* **357**, 695–697 (1992).
- Schreiber, S. L. & Crabtree, G. R. *Immun. Today* **13**, 136–142 (1992).
- McCaifrey, P. G., Perrino, B. A., Soderling, T. R. & Rao, A. J. *biol. Chem.* **268**, 3747–3752 (1993).
- Liu, J. *Immun. Today* **14**, 290–295 (1993).
- Nair, A. P. K. et al. *Molec. cell. Biol.* **9**, 1183–1190 (1989).
- Diamantis, I. D., Nair, A. P. K., Hirsch, H. H. & Moroni, C. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9299–9302 (1989).
- Nair, A. P. K., Hirsch, H. H. & Moroni, C. *Oncogene* **7**, 1963–1972 (1992).
- Hirsch, H. H., Nair, A. P. K. & Moroni, C. *J. exp. Med.* **178**, 403–411 (1993).
- Shaw, G. & Kamen, R. *Cell* **46**, 659–667 (1986).
- Erb, P., Grogg, D., Troxler, M., Kennedy, M. & Fluri, M. J. *Immun.* **144**, 790–795 (1990).
- Wodnar-Filipowicz, A. & Moroni, C. *Proc. natn. Acad. Sci. U.S.A.* **87**, 777–781 (1990).

- Campbell, H. D., Ymer, S., Fung, M. & Young, I. G. *Eur. J. Biochem.* **150**, 297–304 (1985).
- Cockerill, P. N., Shannon, M. F., Bert, A. G., Ryan, G. R. & Vadas, M. A. *Proc. natn. Acad. Sci. U.S.A.* **90**, 2466–2470 (1993).
- Wodnar-Filipowicz, A., Heusser, C. & Moroni, C. *Nature* **339**, 150–152 (1989).
- Abrams, J. S. & Pearce, M. K. *J. Immun.* **140**, 131–137 (1988).
- Ball, P. E., Conroy, M. C., Heusser, C., Davis, J. M. & Conscience, J. F. *Differentiation* **24**, 74–78 (1983).
- Karasuyama, H. & Melchers, F. *Eur. J. Immun.* **18**, 97–104 (1988).
- Gough, N. M. et al. *Nature* **309**, 763–767 (1984).
- Kelso, A. & Gough, N. M. *Growth Factors* **1**, 165–177 (1989).
- Noma, Y. et al. *Nature* **319**, 640–646 (1986).
- Van Snick, J. et al. *Eur. J. Immun.* **18**, 193–197 (1988).
- Yokota, T. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 68–72 (1985).
- Andrejauskas, E. & Moroni, C. *EMBO J.* **8**, 2575–2581 (1989).
- Blochlinger, K. & Diggelmann, H. *Molec. cell. Biol.* **4**, 2929–2931 (1984).

ACKNOWLEDGEMENTS. We thank J. Garcia-Sanz, W. Keller and J. Nakagawa for discussion and for comments on the manuscript, and H.-P. Senn for advice. This work was supported by a grant from the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung.

A two-component system that regulates an osmosensing MAP kinase cascade in yeast

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In the prokaryotic two-component signal transduction systems, recognition of an environmental stimulus by a sensor molecule results in the activation of its histidine kinase domain and phosphorylation of a histidine residue within that domain^{1–3}. This phosphate group is then transferred to an aspartate residue in the receiver domain of a cognate response regulator molecule, resulting in the activation of its output function. Although a few eukaryotic proteins were identified recently that show sequence similarity to the prokaryotic sensors or response regulators, it has not been clear whether they constituted a part of a 'two-component' system^{4–7}. Here we describe a two-component system in *Saccharomyces cerevisiae* that regulates an osmosensing MAP kinase cascade^{8,9}.

To study the physiological function of the *S. cerevisiae* PTP2 tyrosine phosphatase, which is dispensable for vegetative growth^{10–12}, we previously isolated mutants that require expression of PTP2 for survival¹³. One of the three genes thus identified, PTC1, encodes a protein serine/threonine phosphatase type 2C (PP2C)¹³. *ptc1 ptp2* double mutants show a synthetic growth defect, suggesting that PTC1 and PTP2 are involved in a common signal pathway. Mutations that belong to the other two complementation groups (*ypd1* and *ypd2* for tyrosine phosphatase dependent: groups B and A in ref. 13, respectively) were lethal by themselves but their lethality could be suppressed by the overexpression of PTP2. YPD2 encoded a protein of 1,220 amino acids with significant sequence similarity to bacterial sensor molecules. Because YPD2 was found to be the same as

the recently cloned *SLN1* gene⁶, we refer to YPD2 as *SLN1*. The *SLN1* protein (Sln1p) belongs to a subtype of sensors that contain both a histidine kinase domain and a receiver domain in the same protein (Fig. 1c). Gene disruption in diploid cells and analysis of the resulting tetrads indicated that the *sln1Δ* mutation was lethal both on rich (YEPD) and poor (SD) media (data not shown), but the lethality was suppressed when PTP2 was overexpressed. Thus, *sln1Δ* mutant cells that carry a plasmid containing a *P_{GAL1}-PTP2* promoter fusion gene grow normally in media containing galactose, but not in the presence of glucose (Fig. 2, row 1). Introduction of a plasmid containing *SLN1*⁺ into the *sln1Δ* [*P_{GAL1}-PTP2*] mutants eliminates galactose dependency (Fig. 2, row 2).

On the basis of analogy with the prokaryotic homologues, amino-acid residues His 576 and Asp 1,144 are potential sites of phosphorylation in Sln1p (Fig. 1c). To test whether phosphorylation at these residues is essential for Sln1p function, unphosphorylatable mutants were constructed that changed His 576 to Gln (*sln1^{HQ}*), or Asp 1,144 to Asn (*sln1^{DN}*). Expression of either *sln1^{HQ}* or *sln1^{DN}* failed to complement the *sln1Δ* null mutation (Fig. 2, rows 3 and 4), but coexpression of *sln1^{HQ}* and *sln1^{DN}* in the same cell complemented *sln1Δ* (Fig. 2, row 5). These results are consistent with Sln1p being a histidine kinase and that an intermolecular transfer of a phosphate group occurs between His 576 and Asp 1,144.

To identify elements downstream of Sln1p in the signal transduction pathway, we isolated both multicopy suppressor genes and extragenic suppressor mutants of the *sln1* lethality. A screening for multicopy suppressor genes of *sln1Δ* yielded the PTC3 gene and the anticipated PTP2 and *SLN1* genes. The PTC3 gene was previously isolated as the third yeast homologue of PP2C¹³. Furthermore, overexpression of PTC1 also suppressed *sln1Δ*, which is consistent with our earlier observations indicating that Ptp2p and Ptc1p function in the same signal transduction pathway¹³. Figure 2 (rows 6–10) demonstrates that overexpression of the PTP2, PTC1 or PTC3 phosphatase gene, under the control of the strong constitutive ADHI promoter, suppresses the *sln1Δ* lethality. These results suggest that the disruption of *SLN1* causes lethality through the excessive phosphorylation of certain proteins in a signal transduction pathway, and that this phosphorylation is eliminated by overexpression of the protein

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phosphatases Ptp2p, Ptc1p or Ptc3p. However, overexpression of *PTP1* did not suppress the *sln1Δ* lethality, indicating a functional difference between Ptp2p and Ptp1p (Fig. 2, row 7).

Extragenic suppressor mutants were isolated from either *sln1Δ* or *sln1^{ts}* strains. These recessive suppressor mutations fell into one of four complementation groups, *ssk1* to *ssk4* (suppressor of sensor kinase). Nucleotide sequence determination indicated

that *SSK3* was identical to *HOG1*, which encodes a member of the MAP kinase family, and that *SSK4* was identical to *PBS2*, which encodes a MAP-kinase kinase^{8,9}. Both Hog1p and Pbs2p have been implicated in an osmosensing signal transduction pathway, and a rapid, *PBS2*-dependent tyrosine phosphorylation of Hog1p occurs in response to increases in extracellular osmolarity^{9,14}. In contrast, *SSK1* encoded a hitherto un-

FIG. 1 a, Amino-acid sequence of Ssk1p deduced from the nucleotide sequences. Bold type, conserved receiver domain; underline, potential phosphorylation site (Asp 554). The nucleotide sequence of *SSK1* has been deposited in the GenBank database under the accession number L26523. b, A comparison of the receiver domain sequences of Ssk1p and Sln1p with their bacterial homologues. The amino-acid residues that are identical to either Ssk1p or Sln1p are shaded. CheY, *Salmonella typhimurium* CheY¹⁹, BarA, *Escherichia coli* BarA²⁰, RcsC, *E. coli* RcsC²¹. c, Schematic representation of Sln1p and Ssk1p. Putative His and Asp phosphorylation sites are indicated. TM, transmembrane segment. METHODS. A pair of conditionally lethal *sln1Δ* strains, TM181 (*MATa leu2 ura3 trp1 sln1::hisG* + pSSP25) and TM182 (*MATa leu2 ura3 his3 sln1::hisG* + pSSP25) were constructed by tetrad dissection of diploid cells after the disruption of one copy of the *SLN1* gene with *hisG* as described previously^{13,22}. pSSP25 is a centromeric plasmid that carries the *P_{GAL1}-PTP2* promoter-fusion construct and the *URA3* marker¹³. Spontaneously arising galactose-independent revertants (extragenic suppressor mutants of *sln1*) of TM181 and TM182 were isolated on SC-Ura plates. Extragenic suppressor mutants were also isolated from strains that carry the temperature-sensitive *sln1-ts4* allele (our unpublished data). Pairwise crosses of recessive suppressor mutants revealed that there were four complementation groups (*ssk1* through *ssk4*). To clone the *SSK1* gene, an *ssk1* mutant strain was transformed with a YEp13-based genomic library, and transformants that gained galactose-dependence were screened by replica plating.

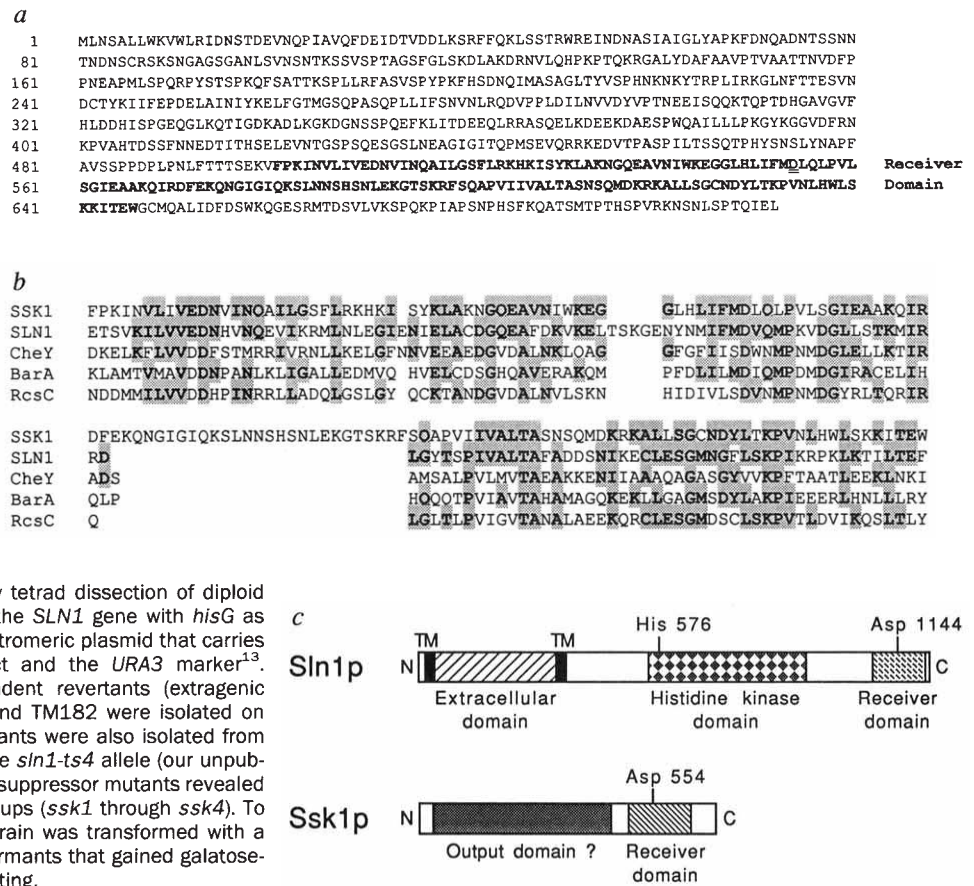
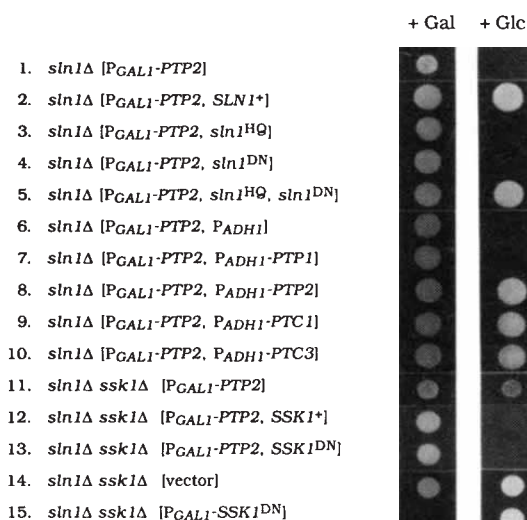


FIG. 2 Characterization of *sln1* and *ssk1* mutants. Genes in square brackets are plasmid-borne. About 1.5×10^4 cells were spotted on appropriate plates containing either galactose or glucose, and plates were incubated at 30 °C for 2 days.

METHODS. The *sln1^{HQ}*, *sln1^{DN}* and *SSK1^{DN}* alleles were constructed by site-directed mutagenesis using sequential PCR. The *SLN1* gene was cloned in pRS415 (*LEU2* marker) and pRS414 (*TRP1* marker) to give rise to pPD2142 and pPD2146, respectively²³. The appropriate segments of pPD2142 and pPD2146 were replaced with the mutant alleles to give rise to pSHQ3 (= *sln1^{HQ} LEU2*) and pSDN2 (= *sln1^{DN} TRP1*), respectively. *PTP1*, *PTP2*, *PTC1* and *PTC3* were individually cloned into the pDBL vector plasmid (*P_{ADH1}*, 2 μ m origin, *LEU2*)²⁴ under the control of the *ADH1* promoter to give rise to pDBT1, pDBT2, pDBC1 and pDBC3, respectively. The *SSK1* gene was cloned in pRS415 to construct pSSK1222. pSSN3 is a derivative of pSSK1222 that carries the *SSK1^{DN}* allele. The *P_{GAL1}-SSK1^{DN}* promoter-fusion gene was inserted into pRS413 (*HIS3* marker) to construct pGSSN1. These plasmids were transformed into one of the following recipient strains: TM181 (*MATa leu2 ura3 trp1 sln1::hisG* + pSSP25), TM187 (*MATa leu2 ura3 trp1 sln1::hisG ssk1::TRP1* + pSSP25) and TM205 (*MATa leu2 ura3 his3 sln1::LEU2 ssk1::URA3*). The yeast strain in each row is as follows: 1, TM181; 2, TM181 + pPD2146; 3, TM181 + pSHQ3; 4, TM181 + pSDN2; 5, TM181 + pSHQ3 + pSDN2; 6, TM181 + pDBL; TM181 + pDBT1; 8, TM181 + pDBT2; 9, TM181 + pDBC1; 10, TM181 + pDBC3; 11, TM187; 12, TM187 + pSSK1222; 13, TM187 + pSSN3; 14, TM205 + pRS413; and 15, TM205 + pGSSN1. The strains in rows 1–13 were grown in SGal medium that was selective for the plasmids before spotting. The strains in rows 14 and 15 were precultured in SC-His medium.



described protein. The predicted amino-acid sequence of Ssk1p contains a receiver domain homologous to the bacterial response regulator molecules (Fig. 1). The *sln1Δssk1Δ* double mutants are viable even in the absence of *PTP2* overexpression, indicating that *ssk1Δ* suppresses the *sln1Δ* lethality (Fig. 2, row 11). The *ssk1Δ* mutation by itself had no obvious phenotype including osmosensitivity (data not shown).

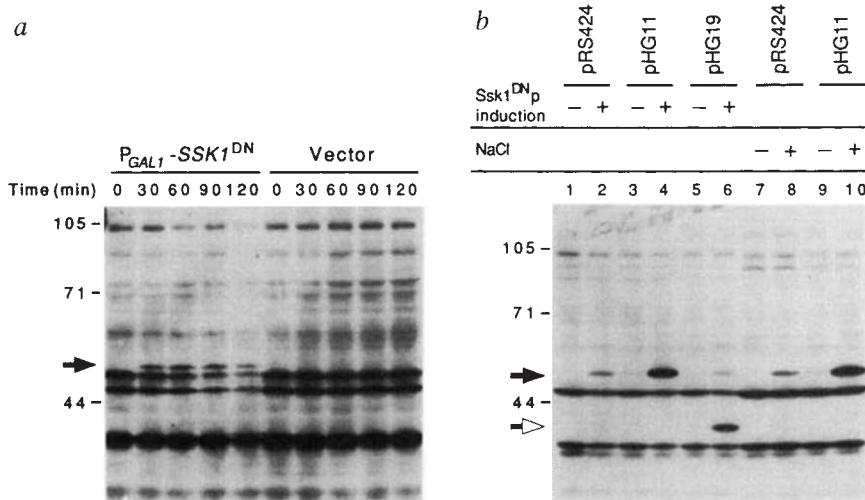
Because Ssk1p is probably unphosphorylated in *sln1Δ* cells, the unphosphorylated Ssk1p is expected to cause lethality. Thus, expression of an unphosphorylatable mutant Ssk1p might also

be lethal in the *sln1Δ* background. To test this prediction, the expected phosphorylation site, Asp 554, was mutated to Asn (*SSK1^{DN}*). Introduction of a plasmid carrying either *SSK1* or *SSK1^{DN}* into the galactose-independent *sln1Δ ssk1Δ* [*P_{GAL1}-PTP2*] mutant strain converted it to galactose-dependent (Fig. 2, rows 11–13). Because *SSK1* and *SSK1^{DN}* complemented the *ssk1Δ* mutation equally well, it was concluded that the unphosphorylated Ssk1p is a functional form.

We then examined the hypothesis that the *sln1* mutation causes overphosphorylation of certain proteins on tyrosine resi-

FIG. 3 **a**, Tyrosine-phosphorylated 50K protein (pp50, indicated by arrow) induced by expression of Ssk1^{DN}p. The yeast strains TM205 + pGSSN1 (*P_{GAL1}-SSK1^{DN}*) and TM205 + pRS413 (Vector) were grown in a raffinose medium, and induced for the expression of Ssk1^{DN}p by the addition of galactose. Tyrosine-phosphorylated proteins were detected by immunoblotting. **b**, Demonstration that pp50 is encoded by *HOG1*. The level of pp50 induced by the expression of Ssk1^{DN}p is elevated when the *HOG1* gene is carried on a multicopy plasmid pHG11 (lanes 1–4). When a multicopy plasmid containing a C-terminal truncated *HOG1* gene (pHG19) was used, a smaller protein was tyrosine phosphorylated (lanes 5 and 6, indicated by empty arrow). The size of the pp50 is identical to the tyrosine-phosphorylated Hog1p induced by salt treatment (lanes 7–10). Yeast strains used in each lane are as follows: 1 and 2, TM210 (*MAT^a leu2 ura3 trp1 his3 sln1::LEU2 ssk1::URA3*) + pGSSN1 + pRS424; 3 and 4, TM210 + pGSSN1 + pHG11; 5 and 6, TM210 + pGSSN1 + pHG19; 7 and 8, TM100 (*MAT^a leu2 ura3 trp1*) + pRS424; 9 and 10, TM100 + pHG11.

METHODS. **a** and **b**, Lanes 1–6, yeast cells were grown in Sraf media (0.67% yeast nitrogen base, 2% raffinose, with appropriate drop-out) at 30 °C. When the OD₆₀₀ of the cultures reached 0.3, galactose was added to a final concentration of 2.5%. Cells were collected at 0 h (**b**, lanes 1, 3 and 5) or 1 h (**b**, lanes 2, 4 and 6) after the addition of galactose. **b**, Lanes 7–10, yeast cells were precultured in SC selective medium, diluted and grown in YEPD for 2 h, and at A₆₀₀ = 0.3, NaCl was added to a final concentration of 0.4 M. Cells were collected 0 min (lanes 7 and 9) or 10 min (lanes 8 and 10) after the addition of NaCl. Cells were suspended in SDS-loading dye, immediately boiled for 4 min,



and resolved by 10% SDS-PAGE. Each lane was loaded with an equivalent amount of protein corresponding to ~0.5 A₆₀₀ units of cells. Proteins were transferred to a nitrocellulose membrane, and the blot was incubated sequentially with monoclonal anti-phosphotyrosine antibody 4G10 (0.1 µg ml⁻¹), rabbit anti-mouse immunoglobulin-peroxidase conjugate, and ECL reagent (Amersham). pHG11 was constructed by insertion of the *HOG1* gene into the multicopy plasmid vector pRS424 (*TRP1* marker). pHG19 was constructed from pHG11 by truncating the *HOG1* gene at a *Pst*I site, resulting in the elimination of the C-terminal 50 amino acids and addition of 4 amino acids derived from the vector sequence.

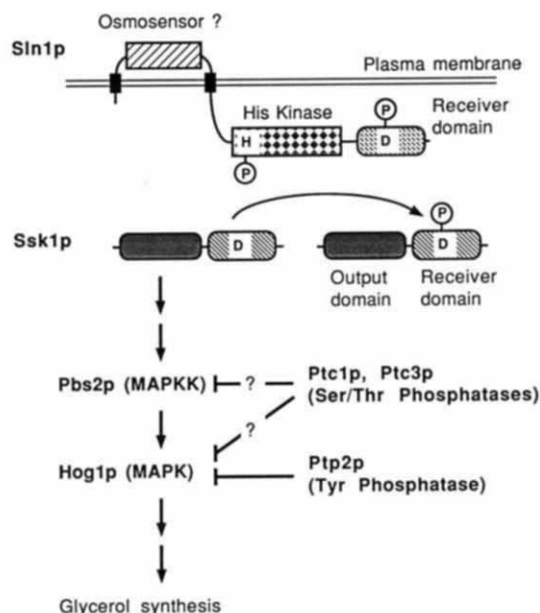


FIG. 4 A possible model of the *SLN1-SSK1* 'two-component' signal transduction system. Sln1p may be a sensor for extracellular osmolarity. When Sln1p is activated by an environmental signal (perhaps low osmolarity), its histidine kinase autophosphorylates (or homo-trans-phosphorylates) the His 576 residue. The phosphate group is then transferred to the conserved aspartate residue in the receiver domains of Sln1p (Asp 1,144) and Ssk1p (Asp 554). The unphosphorylated form of Ssk1p transduces a signal that stimulates the *PBS2-HOG1* MAP kinase cascade. Thus, disruption of *SLN1* leads to a constitutive activation of the MAP kinase cascade, which in turn results in abnormal and deleterious osmoregulatory responses, for example an excessive accumulation of cytosolic glycerol²⁵. Overexpression of the *PTP2* tyrosine phosphatase rescues the *sln1* mutants by reducing the level of *HOG1* tyrosine phosphorylation. The overexpression of *PTC1* or *PTC3* protein serine/threonine phosphatase also suppresses the lethality of *sln1Δ* probably by dephosphorylating (and thus inactivating) Hog1p and/or Pbs2p. The synthetic lethality of *ptp2* and *ptc1* also supports this conclusion¹³. Although Sln1p–Ssk1p is clearly involved in the activation of the osmosensing signal transduction pathway, the lack of osmosensitivity of *ssk1* mutants might suggest the presence of an alternative osmosensor.

dues. A *sln1Δ ssk1Δ* [$P_{GALI-SSK1}^{DN}$] mutant strain grew normally in glucose medium, but when transferred to galactose medium, induction of the $P_{GALI-SSK1}^{DN}$ fusion gene resulted in loss of viability (Fig. 2, rows 14 and 15). Induction of $Ssk1^{DN}$ p also resulted in a rapid appearance of a 50K tyrosine-phosphorylated protein (pp50) (Fig. 3a). Because *hog1* mutations suppress the lethal phenotype of *sln1*, and the *HOG1* MAP kinase is tyrosine phosphorylated on high-salt treatment⁹, we tested the possibility that pp50 is encoded by *HOG1*. To this end, we inserted the *HOG1* gene into the multicopy vector pRS424 (ref. 15) to generate pHG11. The induction of $Ssk1^{DN}$ p synthesis in *sln1Δ ssk1Δ* [$P_{GALI-SSK1}^{DN}$] mutant strain that carries pHG11 resulted in a much higher level of tyrosine phosphorylation than the same mutant strain with pRS424 (Fig. 3b, lanes 2 and 4). Treatment of yeast cells with 0.4 M NaCl induced tyrosine phosphorylation of a protein that is indistinguishable in size from pp50 (Fig. 3b, lanes 7 and 8), whereas a similar treatment of the yeast strain carrying pHG11 resulted in a higher level of tyrosine-phosphorylated protein (Fig. 3b, lanes 9 and 10). Furthermore, when a plasmid containing a *HOG1* gene with a C-terminal truncation (pHG19) was used, a smaller protein was tyrosine phosphorylated on the induction of $Ssk1^{DN}$ p synthesis (Fig. 3b, lanes 5 and 6). These results indicated that the expression of *SSK1* in the absence of *SLN1* induced tyrosine phosphorylation of the *HOG1* MAP kinase.

We have identified the first, to our knowledge, complete pair of a 'two-component' signal transduction system in eukaryotic cells. A possible mechanism of the *SLN1-SSK1* signal transduction pathway is shown in Fig. 4. *Sln1*p histidine kinase might be a transmembrane osmosensor. In high-osmolarity media, the inactive *SLN1* histidine kinase allows the unphosphorylated *Ssk1*p to activate the *PBS2-HOG1* MAP kinase cascade. The *PTP2*, *PTC1*, and perhaps *PTC3* protein phosphatases may regulate this pathway by dephosphorylating *Hog1*p and possibly *Pbs2*p. In low-osmolarity media, the active *SLN1* histidine kinase represses the activation of the *PBS2-HOG1* kinase cascade

through phosphorylation of *Ssk1*p. Finally, our results imply that *Ptp2*p is a specific phosphatase for the *HOG1* MAP kinase. Recently, a family of dual-specificity protein phosphatases that are remotely related to the PTP gene family have been identified as specific MAP-kinase phosphatases¹⁶⁻¹⁸. Thus, it appears that there are two separate types of MAP-kinase phosphatases that differ both in structure and function. □

Received 9 March; accepted 15 April 1994.

1. Parkinson, J. S. & Kofoid, E. C. A. Rev. Genet. **26**, 71-112 (1992).
2. Stock, J. B., Lukat, G. S. & Stock, A. M. A. Rev. biophys. Chem. **20**, 109-136 (1991).
3. Bourret, R. B., Borkovich, K. A. & Simon, M. I. A. Rev. Biochem. **60**, 401-441 (1991).
4. Schneider-Poetsch, H. A. W., Braun, B., Marx, S. & Schaumburg, A. FEBS Lett. **281**, 245-249 (1991).
5. Chang, C., Kwok, S. F., Bleecker, A. B. & Meyerowitz, E. M. Science **262**, 539-544 (1993).
6. Ota, I. M. & Varshavsky, A. Science **262**, 566-569 (1993).
7. Brown, J. L., North, S. & Bussey, H. J. Bact. **175**, 6908-6915 (1993).
8. Boguslawski, G. & Polazzi, T. Proc. natn. Acad. Sci. U.S.A. **84**, 5848-5852 (1987).
9. Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. & Gustin, M. C. Science **259**, 1760-1763 (1993).
10. Ota, I. & Varshavsky, A. Proc. natn. Acad. Sci. U.S.A. **89**, 2355-2359 (1992).
11. James, P., Hall, B. D., Whelen, S. & Craig, E. A. Gene **122**, 101-110 (1992).
12. Guan, K., Deschenes, R. J. & Dixon, J. E. J. biol. Chem. **267**, 10024-10030 (1992).
13. Maeda, T., Tsai, A. Y. M. & Saito, H. Molec. cell. Biol. **13**, 5408-5417 (1993).
14. Boguslawski, G. J. gen. Microbiol. **138**, 2425-2432 (1992).
15. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. & Hieter, P. Gene **110**, 119-122 (1992).
16. Sun, H., Charles, C. H., Lau, L. F. & Tonks, N. K. Cell **75**, 487-493 (1993).
17. Ward, Y. et al. Nature **367**, 651-654 (1994).
18. Doi, K. et al. EMBO J. **13**, 61-70 (1994).
19. Stock, A. M., Koshland, D. E. J. & Stock, J. Proc. natn. Acad. Sci. U.S.A. **82**, 7989-7993 (1985).
20. Nagasawa, S., Tokishita, S., Aliba, H. & Mizuno, T. Molec. Microbiol. **6**, 799-807 (1992).
21. Stout, V. & Gottesman, S. J. Bact. **172**, 659-669 (1990).
22. Alani, E., Cao, L. & Kleckner, N. Genetics **118**, 541-545 (1987).
23. Sikorski, R. S. & Hieter, P. Genetics **122**, 19-27 (1989).
24. Milne, G. T. & Weaver, D. T. Genes Dev. **7**, 1755-1765 (1993).
25. Mager, W. H. & Varela, J. C. S. Molec. Microbiol. **10**, 253-258 (1993).

ACKNOWLEDGEMENTS. We thank T. Thai for technical assistance, N. X. Krueger, P. O'Grady, and M. Streuli for comments, and F. Winston, K. Dollard, T. Christianson, T. Milne, D. Weaver, R. Kolodner and T. Ernst for yeast strains, plasmids, libraries and oligonucleotides. This work was supported by grants from the Barr Program at the DFCL and the NIH to H.S., and a postdoctoral fellowship from Sandoz to T.M.

Histone H3 amino terminus is required for telomeric and silent mating locus repression in yeast

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HETEROCHROMATIN is a cytologically visible form of condensed chromatin capable of repressing genes in eukaryotic cells. For the yeast *Saccharomyces cerevisiae*, despite the absence of observable heterochromatin, there is genetic and chromatin structure data which indicate that there are heterochromatin-like repressive structures^{1,2}. Genes experience position effects at the silent mating loci and the telomeres, resulting in a repressed state that is inherited in an epigenetic manner. The histone H4 amino terminus is required for repression at these loci^{3,4}. Additional studies have indicated that the histone H3 N terminus is not important for silent mating locus repression^{5,6}, but redundancy of repressive elements at the silent mating loci may be responsible for masking its role. Here we report that histone H3 is required for full repression at yeast telomeres and at partially disabled silent mating loci, and that the acetylable lysine residues of H3 play an important role in silencing.

We tested the role of H3 in telomeric repression by integrating the *URA3* gene at a telomeric locus (Fig. 1a), assayed by sensitivity to 5-fluoro-orotic acid (5-FOA). The *URA3* gene was strongly repressed in wild-type cells (Fig. 1b), as observed previously⁷. In contrast, increasingly larger deletions of the H3 N terminus resulted in increased sensitivity to 5-FOA, indicating a loss of repression of the telomeric *URA3* gene. These data define a domain of residues 4-20 that is required for telomeric repression. Additionally, we found that single amino-acid substitutions in the non-basic silencing domain (R2)⁸ of histone H4 also resulted in derepression of telomeric *URA3* (Fig. 1c), as was previously found⁴ for substitutions in the basic silencing domain R1. These results are in contrast to a deletion of the histone H2B N terminus, which caused no increase in 5-FOA sensitivity (Fig. 1c). Previous data have also demonstrated no significant effect of H2A or H2B N-terminal deletions on repression at the silent mating loci³.

Because mutations in both H3 and H4 caused full sensitivity to 5-FOA, we examined the *URA3* transcript levels by an RNase protection assay to determine relative expression levels. The *URA3* transcript was detected at increased levels in the H3 N-terminal deletion strains relative to the wild-type strain (Fig. 2), but was lower than that in H4 mutant strains. Because the 5-FOA assay is a measure of the percentage of cells in which the *URA3* gene is repressed, the combination of these two experiments indicates that mutations in H3 cause a loss of repression in nearly all cells in a population, but that the expression level per cell is lower than that observed in H4 mutant cells.

The N-terminal tails of the histones possess highly conserved lysine residues which are subject to post-translational

acetylation⁹. Transcriptionally active regions generally consist of hyperacetylated histones, whereas silent regions have reduced levels of acetylation^{10,11}, although no causal relationship has been established. To analyse the role of the H3 lysine residues, we tested the effect of single amino-acid substitutions of these residues on telomeric repression. Lysines were changed to argin-

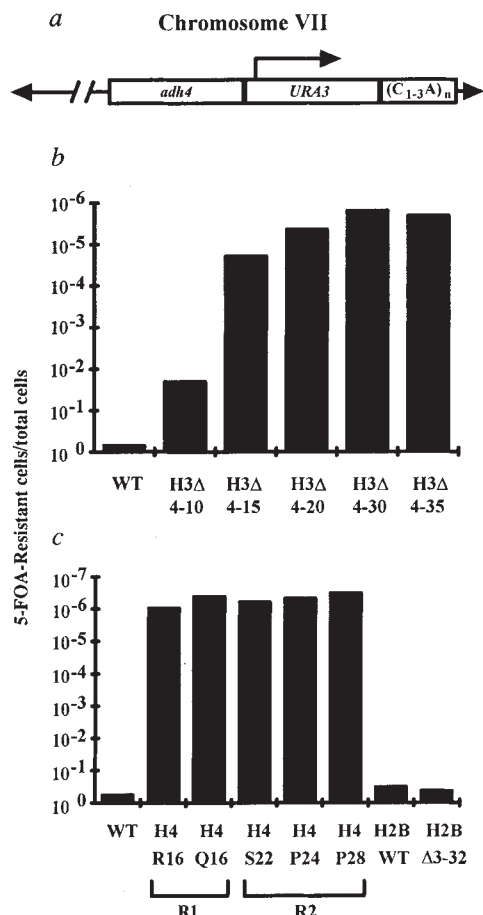


FIG. 1 Mutations in histones H3 and H4 result in the loss of telomeric repression. **a**, Schematic representation of the *URA3* gene integrated at the *adh4* locus adjacent to the telomere on the left arm of chromosome VII. The arrow above the *URA3* gene indicates the orientation of transcription. C_{1-3A} refers to the telomeric repeat sequence derived from the integrating vector. **b**, Histone mutant strains with a copy of the *URA3* gene integrated at the telomere on the left arm of chromosome VII were analysed for the ability to grow on media containing 5-fluoro-orotic acid (5-FOA). Expression of the *URA3* gene results in inviability in the presence of 5-FOA due to conversion of 5-FOA into toxic 5-fluorouracil¹⁵. Strains possess deletions of the indicated residues of the N terminus of histone H3⁶. **c**, Strains were analysed as in **b**, possessing single amino-acid substitutions in either domain R1 or R2 of the histone H4 N terminus^{8,14}, or possessing an N-terminal deletion of histone H2B¹⁶. The H4 mutations are R16 (lysine to arginine at position 16), Q16 (lysine to glutamine at position 16), S22 (leucine to serine at position 22), P24 (aspartate to proline at position 24), and P28 (glycine to proline at position 28). The fraction of cells able to grow in the presence of 5-FOA was determined (relative to non-5-FOA containing media), and the data are graphed logarithmically. WT, Wild-type strain.

METHODS. The *URA3* gene was integrated at the *adh4* locus adjacent to the right telomere as previously described⁷. Integrations were done directly into H3 mutant strains, whereas H4 mutations were introduced into the H3 parent strain (JTY102TU:RMY102T⁶ with the *adh4::URA3* integration) by a plasmid shuffle technique⁶. Histone H2B mutant strains were constructed by replacing the plasmid carrying *HTB2* (histone H2B) in strain TSY155¹⁶ with an *HTB2* plasmid possessing a *HIS3* marker, followed by integration of *URA3* at *adh4*. 5-FOA assays were as previously described⁷.

ine to mimic the unacetylated state, or to an uncharged residue (glycine or glutamine) to mimic the acetylated state. We found that single-site substitutions at positions 9 and 14 had little effect on telomeric repression (Fig. 3). In contrast, substitution of 3 or 4 lysine residues simultaneously (at positions 9, 14, 18 and 23) resulted in decreased 5-FOA resistance levels to about 10⁻¹.

TABLE 1 Histone H3 N-terminal deletions derepress weakened silent mating loci

Strain	MAT	Histone H3	Histone H4	SIR1	Mating efficiency
GFY3000	<i>a</i>	+	+	+	1.0
XLY409 <i>a</i>	<i>a</i>	+	H4Q16	+	0.68
GFY3430	<i>a</i>	H3Δ4-30	+	+	0.58
XLY410 <i>a</i>	<i>a</i>	H3Δ4-30	H4Q16	+	2.0 × 10 ⁻⁵
RMY200	<i>a</i>	+	+	+	1.0
JTY100	<i>a</i>	+	+	<i>sir1-</i>	1.2
RMY430	<i>a</i>	H3Δ4-30	+	+	0.27
JTY102	<i>a</i>	H3Δ4-30	+	<i>sir1-</i>	0.017

MATa strains were constructed by introducing mutations in histone H3⁶ and/or histone H4¹⁴ into the parent strain GFY3000 (G. Fisher-Adams and M.G., unpublished data), by the plasmid shuffle technique⁶. *MATa sir1-* strains were constructed by disrupting the *SIR1* gene in strains RMY200 and RMY430⁶ with the *URA3* gene, using plasmid pJT101. Mating efficiencies were determined by a quantitative mating assay as previously described¹². Each set of mating efficiencies was normalized to the corresponding wild-type mating efficiency. +, Wild-type allele.

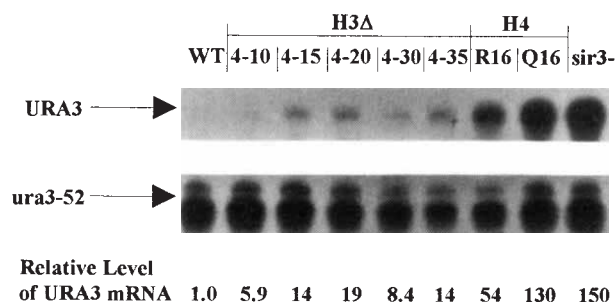
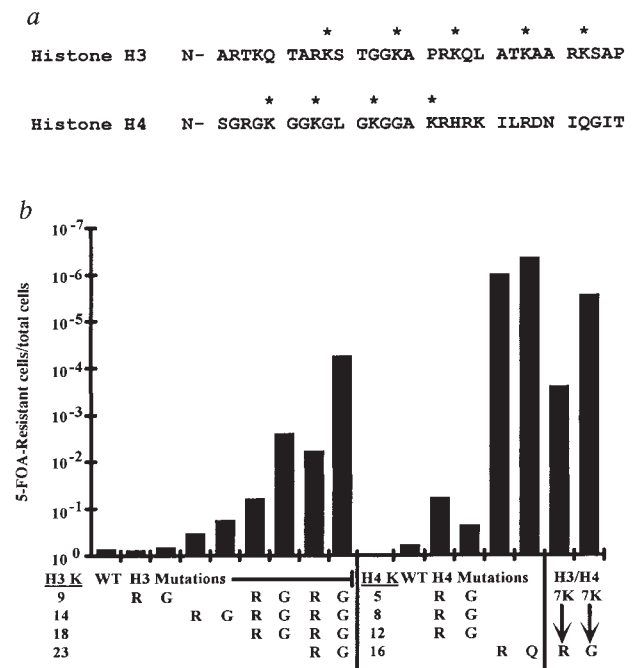


FIG. 2 RNase protection analysis of *URA3* transcripts expressed from a telomeric locus in histone H3 and H4 mutant strains. Transcript levels were measured from RNA preparations from the same strains described in Fig. 1. The *URA3* transcript is produced from the copy of the gene integrated at the left telomere of chromosome VII, whereas the *ura3-52* transcript is the mutant transcript produced at the native *URA3* locus on chromosome V (expressed constitutively). Data shown are from a 48-h exposure (*URA3*) or a 16-h exposure (*ura3-52*) of the same autoradiogram. Low-level *URA3* expression can be visualized in the wild-type strain on long exposures (>48 h; data not shown). Transcript levels were quantified by densitometric scanning using the Apple Color OneScanner, and analysed with the NIH Image 1.49 program. The densitometric values were adjusted using the level of *ura3-52* transcripts to correct for RNA quantification errors between samples (variation between samples was no more than 1.6-fold), and were normalized relative to the value for the wild-type (WT) strain.

METHODS. Total cellular RNA was isolated from the strains in Fig. 1 as previously described¹⁷. The *sir3-* strain was constructed by integrating *URA3* at *adh4* in the strain LJY155 (*sir3::LEU2*; L. Johnson, unpublished data). An RNA probe that hybridized to the *URA3* transcript was generated using the plasmid pJT135 (a PCR-generated fragment of *URA3* from base 210 to 460 cloned into the *EcoRI*-*PstI* sites of pBluescript II KS [+/-]), pJT135 was linearized with *Bam*HI and used for *in vitro* transcription using the RNA Transcription Kit (Stratagene), synthesized in the presence of α-³²P-UTP (800 Ci mmol⁻¹, 40 mCi ml⁻¹; Amersham). RNase protection assays were done using the Ambion RPA II Kit (Ambion, Inc.), according to the kit instructions.

FIG. 3 Mutation of the acetylable lysine residues in histone H3 and H4 derepresses *URA3* at the telomere. *a*, Amino-acid sequence of the first 30 residues of histones H3 and H4. Asterisks over the lysine (K) residues indicate sites of potential acetylation. *b*, Strains expressing mutant forms of histones H3 and H4 (with an integrated copy of *URA3* at the left telomere of chromosome VII) were tested for their ability to survive on media containing 5-FOA, as described in Fig. 1. On the left, strains expressing mutations of the acetylable lysine (K) residues in histone H3⁶ (positions 9, 14, 18 and 23). Lysines were altered to either arginine (R) or glycine (G). In the middle, strains expressing mutations of the acetylable lysine residues in histone H4^{8,16} (positions 5, 8, 12 and 16). Lysines were altered to arginine (R), glycine (G) or glutamine (Q). On the right are two strains which possess substitutions of H3 residues 9, 14, 18 and 23 and H4 residues 5, 8 and 12, changed to either arginine (7K→R) or glycine (7K→G). Strains were constructed and 5-FOA assays done as described in the legend to Fig. 1. WT, Wild type.



10^{-5} , suggesting that the number of modified lysine residues in H3 influences the degree of derepression. In comparison, substitution of the lysine residue at position 16 in histone H4 caused a strong decrease in 5-FOA resistance, as shown previously⁴, but simultaneous substitutions at positions 5, 8 and 12 had at most a minor effect. To test the possibility that acetylable lysines in H3 and H4 function synergistically, we combined the substitution of four lysines in H3 with similar substitutions at positions 5, 8 and 12 in H4 (7K→R, G). These strains exhibited reduced levels of 5-FOA resistance compared with the H3 substitutions alone, indicating that the first three lysines in H4 do contribute to the repressed state, and that H3 and H4 acetylation sites function synergistically.

Although both substitutions to arginine or glycine resulted in some derepression, we consistently observed increased 5-FOA sensitivity with the glycine substitutions compared with the corresponding arginine substitutions (30–100-fold differences). These results suggest that histone acetylation causes derepression at the telomere. We cannot rule out the possibility, however, that the uncharged substitutions produced a stronger effect due to structural defects in the N terminus imposed by the particular substitution, rather than being due to changes in the electrostatic charge.

To determine if functional redundancy of the silent mating loci is responsible for masking the effect of H3 mutations, we tested the effect of H3 N-terminal deletions on silent mating loci

impaired by weak silencer mutations. We combined the H3 N-terminal deletion $\Delta 4$ –30 with the H4Q16 mutation in a *MATa* background to determine the effect on repression at *HMR* (derepression of *HMRa* causes a non-mating phenotype in *MATa* cells). Neither H3 nor H4 mutation alone had an effect at *HMR* (Table 1; shown previously¹² for H4), but the combination of the two mutations resulted in complete derepression of *HMR*. Similarly, we tested the effect of the H3 N-terminal deletion $\Delta 4$ –30 at *HML* in a *MATa sir1* strain. The *sir1* mutation had no effect on mating efficiency whereas the H3 $\Delta 4$ –30 mutation had a very small effect, as shown previously^{6,13}, but the combination of the two resulted in a 16-fold decrease in mating efficiency (relative to H3 $\Delta 4$ –30 alone), indicating partial derepression of *HML*. Although mutations in H3 do not impose as severe a silencing defect as do mutations in H4, H3 is clearly required for full repression at the silent mating loci.

The precise mechanism by which the histone H3 and H4 N termini mediate heterochromatic repression remains unclear, but genetic¹⁴ and biochemical data (Hecht and M.G., unpublished results) indicate that they may function by interacting with accessory silencer proteins Sir3 and Sir4. Such associations may lead to the assembly of a multiprotein complex which aids chromatin condensation and inhibits the access of transcription factors. These interactions could be modulated by histone H3 and H4 acetylation (ref. 11 and this work), limiting the silenced state to select regions in the genome. □

Received 7 February; accepted 11 April 1994.

- Laurenson, P. & Rine, J. *Microbiol. Rev.* **56**, 543–560 (1992).
- Thompson, J. S., Hecht, A. & Grunstein, M. *Cold Spring Harb. Symp. quant. Biol.* **58**, 247–256 (1993).
- Kayne, P. S. et al. *Cell* **55**, 27–39 (1988).
- Aparicio, O. M., Billington, B. L. & Gottschling, D. E. *Cell* **66**, 1279–1287 (1991).
- Morgan, B. A., Mittman, B. A. & Smith, M. M. *Molec. cell. Biol.* **11**, 4111–4120 (1991).
- Mann, R. K. & Grunstein, M. *EMBO J.* **11**, 3297–3306 (1992).
- Gottschling, D. E., Aparicio, O. M., Billington, B. L. & Zakian, V. A. *Cell* **63**, 751–762 (1990).
- Johnson, L. M., Fisher-Adams, G. & Grunstein, M. *EMBO J.* **11**, 2201–2209 (1992).
- van Holde, K. E. *Chromatin* (Springer, Berlin, 1989).
- Turner, B. M., Birley, A. J. & Lavender, J. *Cell* **69**, 375–384 (1992).

- Braunstein, M., Rose, A. B., Holmes, S. G., Allis, C. D. & Broach, J. R. *Genes Dev.* **7**, 592–604 (1993).
- Thompson, J. S., Johnson, L. M. & Grunstein, M. *Molec. cell. Biol.* **14**, 446–455 (1994).
- Pillus, L. & Rine, J. *Cell* **59**, 637–647 (1989).
- Johnson, L. M., Kayne, P. S., Kahn, E. S. & Grunstein, M. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6286–6290 (1990).
- Boeke, J. D., LaCroute, F. & Fink, G. R. *Molec. Gen. Genet.* **197**, 345–346 (1984).
- Schuster, T., Han, M. & Grunstein, M. *Cell* **45**, 445–451 (1986).
- Kohrer, K. & Domdey, J. *Meth. Enzym.* **194**, 398–405 (1991).
- Durrin, L. K., Mann, R. K., Kayne, P. S. & Grunstein, M. *Cell* **65**, 1023–1031 (1991).

ACKNOWLEDGEMENTS. We thank D. Gottschling, G. Fisher-Adams and R. K. Mann for yeast strain and plasmid contributions. This work was supported by a grant from the NIH. J.S.T. was supported by a USPHS National Research Service Award.

How does a protein fold?

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THE number of all possible conformations of a polypeptide chain is too large to be sampled exhaustively. Nevertheless, protein sequences do fold into unique native states in seconds (the Levinthal paradox). To determine how the Levinthal paradox is resolved, we use a lattice Monte Carlo model in which the global minimum (native state) is known. The necessary and sufficient condition for folding in this model is that the native state be a pronounced global minimum on the potential surface. This guarantees thermodynamic stability of the native state at a temperature where the chain does not get trapped in local minima. Folding starts by a rapid collapse from a random-coil state to a random semi-compact globule. It then proceeds by a slow, rate-determining search through the semi-compact states to find a transition state from which the chain folds rapidly to the native state. The elements of the folding mechanism that lead to the resolution of the Levinthal paradox are the reduced number of conformations that need to be searched in the semi-compact globule ($\sim 10^{10}$ versus $\sim 10^{16}$ for the random coil) and the existence of many ($\sim 10^3$) transition states. The results have evolutionary implications and suggest principles for the folding of real proteins.

The mechanism of protein folding is not understood, despite many studies devoted to this subject¹. The essential question is

how a polypeptide chain is able to fold rapidly, in milliseconds to seconds, to the native state, despite the very large number of conformations that exist for the chain (Levinthal paradox)². To approach this problem, we used a simplified model that consists of a 27-bead self-avoiding chain on a cubic lattice³ (Fig. 1). The native (lowest energy) state can be determined exactly³ and a survey of the folding behaviour of many sequences is possible⁴. In addition, the full phase space density of the system can be obtained and the thermodynamic properties can be calculated as a function of the folding 'reaction coordinate'. The model is sufficiently complex that a resolution of the Levinthal paradox is required for folding: some sequences find the native state in only $\sim 10^7$ Monte Carlo steps even though there are $\sim 10^{16}$ possible conformations (Fig. 3b). Because the lattice model does not include the amino-acid side chains, the process considered here may correspond to the folding of real proteins to the molten globule state; that is, the molten globule, if it has a defined fold, is the native state in the present model.

In the first part of the analysis, 200 sequences with random interactions were generated and subjected to Monte Carlo folding simulations (Fig. 2)⁴. Of these, 30 chains found the known native state in a short time. These chains correspond to actual protein sequences in the sense that they fold on a reasonable timescale; the remaining sequences do not represent protein sequences because they do not fold rapidly enough, although they do have a unique minimum. These sequences serve as controls. The 30 folding sequences were analysed and compared with the non-folding sequences. Several suggested mechanisms for resolving the Levinthal paradox do not apply to the present model as the features assumed to be responsible for rapid folding are found to be the same for the folding and non-folding sequences. These include a high number of short- versus long-

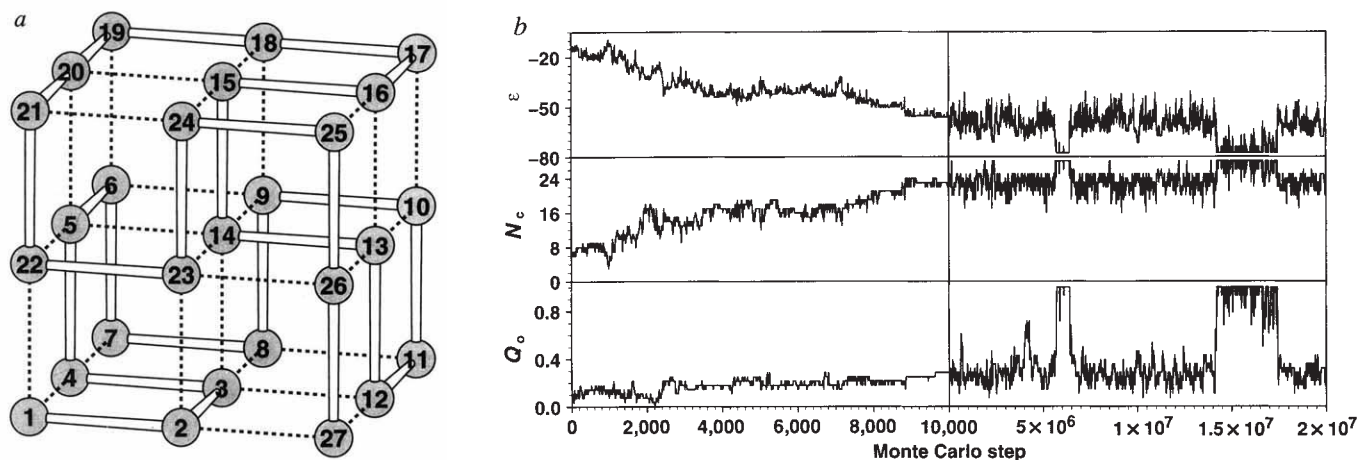


FIG. 1 Lattice model of protein folding. *a*, An example of a compact self-avoiding structure of a chain of 27 monomers (filled numbered circles) with 28 contacts (dashed lines). The structure shown is the native state of the folding random sequence 43 used as an example in this paper. The total energy of a conformation is the sum of contact energies: $E = \sum_{i < j} \Delta(r_i, r_j) B_{ij}$, where r_i are the positions of monomers i , B_{ij} are the contact energies for pairs of monomers i, j , and $\Delta(r_i, r_j)$ is 1 if monomers i and j are in contact and is 0 otherwise; two monomers are in contact if they are not successive in sequence and at unit distance from each other. The values of the B_{ij} are obtained from a gaussian distribution with a mean B_0 and standard deviation σ_B . This particular model for B_{ij} corresponds to a heteropolymer with a random sequence of monomers of many different types whose heterogeneity is measured by σ_B ¹¹. The parameter B_0 is an overall attractive term that emulates the hydrophobic effect observed in globular proteins. A particular sequence is defined by the matrix of contact energies B_{ij} .

The B_{ij} s correspond to the contact energies in real proteins⁴, such as those described by Miyazawa and Jernigan¹⁹. The native conformation is the compact self-avoiding chain with the lowest energy. *b*, Typical trajectory for a folding random sequence ($T=1.3$). Energy, ϵ (in units of $k_B T$ where k_B is the Boltzmann constant); the number of contacts, N_c ; fraction of the number of contacts in common with the native state, Q_0 . The instantaneous values of these quantities are plotted every 10 Monte Carlo (MC) steps in the first part of the trajectory ($\leq 10,000$ MC steps) and every 20,000 steps in the subsequent part. The folding trajectory starts with a random-coil conformation and consists of local MC moves of one or two successive monomers that preserve bond lengths and avoid multiple occupancy of the lattice sites⁴; one MC step corresponds to a move and a test of its acceptance with the Metropolis criterion²⁰. In the first part of the trajectory, $\sim 50\%$ of the MC moves are accepted, whereas only 5–10% of the moves are successful in the subsequent part of the simulation.

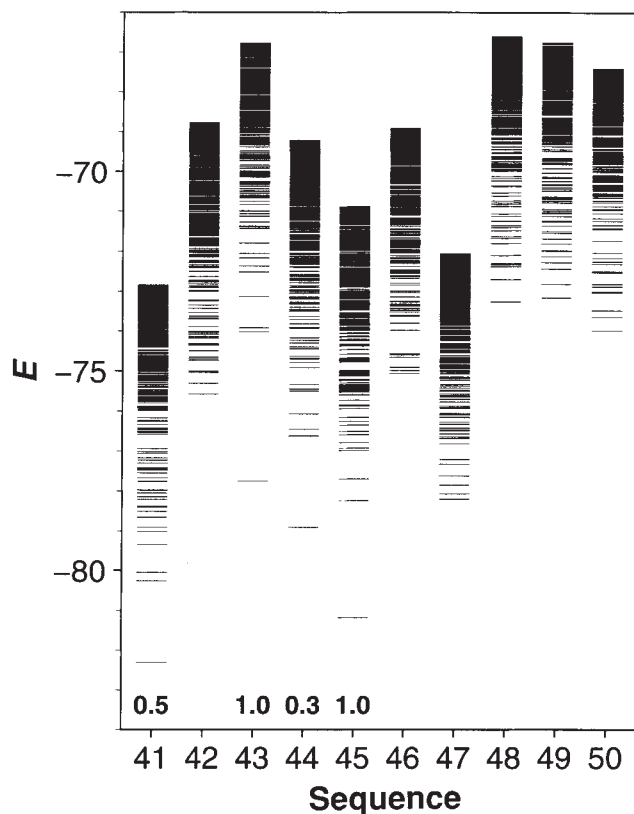


FIG. 2 Energy spectra for 10 folding and non-folding random sequences. The energies of the 400 lowest compact self-avoiding conformations are shown. The native state corresponds to the bottom bar. The numbers below the spectra show the folding tendencies of the corresponding sequences; if no number is given, folding tendency is 0. A sequence folds in a given MC simulation if it finds the native conformation within 50×10^6 MC steps. Folding tendency of a given sequence is defined as the fraction of 10 MC runs that started with a random conformation and reached the native conformation under a given set of conditions. A sequence is a folding sequence if the native conformation is structurally unique and folding tendency is high (≥ 0.4) under conditions where the native structure is thermodynamically stable. A sequence is a non-folding sequence if the folding tendency is 0.0. There are 24 intermediate sequences that we do not consider here. Optimal values for parameters $B_0(-2)$ and $\sigma_B(1)$ were determined by exhaustive sampling of folding tendency as a function of these two parameters⁴. Each sequence is studied at a temperature where the native state has a high probability to be reasonably thermodynamically stable; the native state has a weight larger than 0.2 relative to other compact self-avoiding conformations⁴.

range contacts in the native state⁶, a high content of secondary structure in the native state⁷, a strong correlation between the native contact map and the interaction parameters⁸, and the existence of a high number of low-energy states with near-native conformations³. Moreover, there is no repetitive trapping of the non-folding sequences in the same local minimum, so that the native state cannot be a metastable state⁹. The only significant difference between folding and non-folding sequences is that the native state is at a pronounced energy minimum (Fig. 2). This is the necessary and sufficient condition for a sequence to fold rapidly in the present model.

To explore the mechanism of folding, the density of states was determined as a function of the energy and the reaction coordinate (the number of native contacts) (Fig. 3); a related reaction coordinate, the number of residues in the native conformation, has been used in other models of folding¹⁰. From the density of states, the mean energy, entropy and free energy are calculated (Fig. 4a-c). Above the critical temperature ($T=1$), the energy and entropy decrease smoothly as the chain approaches the native state. The free energy, by contrast, has a maximum corresponding to the transition region that separates the denatured and native states. This barrier, which makes the reaction a cooperative transition, is dominated by the entropic contribution, as can be seen from the temperature dependence of the free energy. Below the critical temperature, the free-energy reaction profile becomes rugged^{10,11} and folding is very slow because the chain is likely to be trapped in one of the many local minima.

The time history of the folding process (Fig. 1b) shows that there is a rapid collapse in about 10^4 Monte Carlo steps to a semi-compact random globule; the number of contacts increases, the energy decreases, whereas the fraction of native contacts remains below 0.3. In this way, the total of $\sim 10^{16}$ random-coil conformations is reduced to $\sim 10^{10}$ random semi-compact globule states. The fast collapse results from a large energy gradient

(Fig. 1b) and the presence of many empty lattice points. In the second stage (Fig. 4d), which is rate-limiting, the chain searches for one of the $\sim 10^3$ transition states. The transition region consists of all states from which the chain folds rapidly to the native state. The transition states are structurally similar to the native state, with 23–26 (80–95%) of the native contacts. Generally, the mean first passage time, τ , for finding any of the n states among the total of N states by a random search which explores r states per unit of time is $N/(n \times r)$. For the present model, $\tau \sim 10^{10}/(10^3 \times 1) = 10^7$ (Fig. 3c), identical to the observed time-scale. This indicates that the rate-limiting stage in folding consists of a random search for a transition state in the semi-compact part of the phase space; a folding 'pathway' is not involved in finding the native state. In the third stage, the chain rapidly (within $\sim 10^5$ Monte Carlo steps) attains the native conformation from any one of the transition states.

The pronounced energy minimum is the necessary condition for the folding of a 27-mer on a lattice because it guarantees that the native state is stable above the critical temperature, where the rearrangements with local unfolding required in the rate-limiting stage are energetically possible. It is also a sufficient condition because a random search of compact globules with random structures can rapidly find a transition state that folds to the stable native state in a short time. Although a non-folding sequence may also fold slowly to its native state above the critical temperature, it would not be stable at that temperature and therefore could not correspond to a real protein.

The size of the search for the native state is greatly reduced when the chain is semi-compact, as it is in real proteins¹². Nevertheless, in the 27-mer model, as in real proteins, a random search of a collapsed globule cannot find the native state in the observed time. It is the existence of a transition region, consisting of a large number of states, that reduces the search time to realistic values. Extrapolation of the folding time to real proteins suggests that the three-stage random-search mechanism could be effective

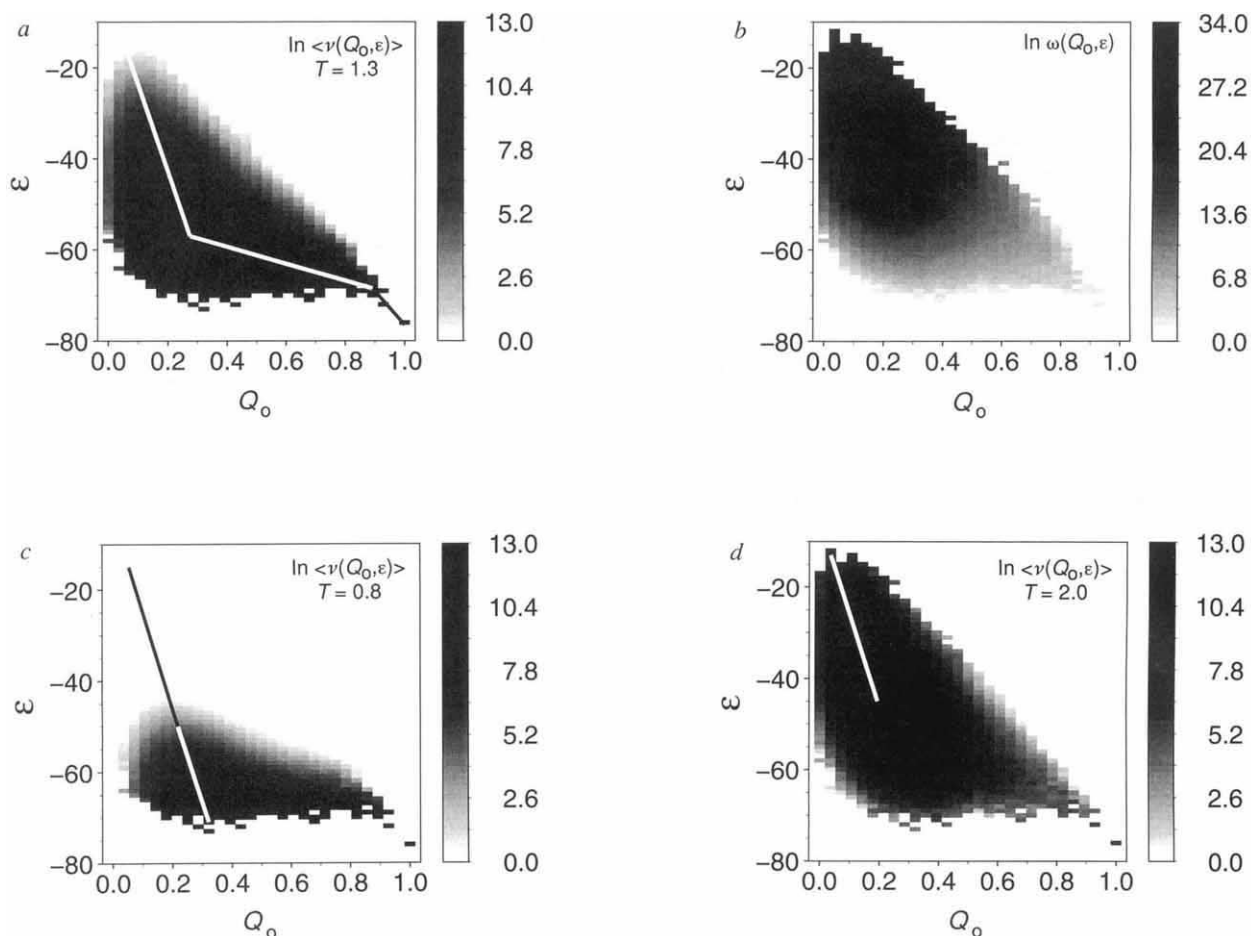


FIG. 3 Density of states for a folding random sequence, as obtained from a long Monte Carlo simulation. *a*, The logarithm of the average occupancy of the (Q_0, ϵ) bins, $\langle v(Q_0, \epsilon) \rangle$, at $T = 1.3$. Each bin spans $1/28 Q_0$ units and 1 energy unit. The occupancy of a bin is the number of Monte Carlo steps that remain or result in any of the conformations corresponding to the bin. The average is calculated from 20 independent MC sampling simulations of 50×10^6 steps each (A.Š., E.S. and M.K., manuscript in preparation). The broken line indicates a typical folding pathway. *b*, The logarithm of the density of states, $\omega(Q_0, \epsilon)$. The density of states is calculated from $\langle v(Q_0, \epsilon) \rangle$ by the use of $\langle v(Q_0, \epsilon) \rangle / \langle v(Q_0 = 1, \epsilon = \epsilon_0) \rangle = \omega(Q_0, \epsilon) / \omega(Q_0 = 1, \epsilon = \epsilon_0) \exp[-\epsilon/(k_B T)] / \exp[-\epsilon_0/(k_B T)]$ where ϵ_0 is the energy of the native state and k_B is the Boltzmann constant set to 1; because $\omega(Q_0 = 1, \epsilon = \epsilon_0) = 1$, the density of states is $\omega(Q_0, \epsilon) = \langle v(Q_0, \epsilon) \rangle / \langle v(1, \epsilon_0) \rangle \exp[-(\epsilon_0 - \epsilon)/(k_B T)]$. The calculation is accurate because thermodynamic equilibrium at the present Q_0, ϵ resolution is achieved, as demonstrated by a small fractional error in $\langle v(Q_0, \epsilon) \rangle$ (less than 10% in the populated parts of the phase space). The summation of $\omega(Q_0, \epsilon)$ over all Q_0 and ϵ results

in about 1.1×10^{16} self-avoiding states; this is in good agreement with the extrapolation of the exact enumerations for shorter chains: $v^{L-1}/12 = 2.2 \times 10^{16}$ where L is the number of monomers, $v = 4.68$ is the average number of monomer states, and division by 12 corrects for symmetry²¹. Semi-compact states are defined as the states with energy less than $-45k_B T$; summation over the appropriate region of $\omega(Q_0, \epsilon)$ yields $\sim 10^{10}$ such states. The transition states correspond to all the states with $0.8 \leq Q_0 < 1$; there are $\sim 10^3$ such states. *c*, The average occupancy of the bins at a low $T (T = 0.8)$ as calculated from $\omega(Q_0, \epsilon)$. The line shows the rapid collapse of a random coil chain into a random semi-compact frozen conformation. *d*, The calculated average occupancy of the bins at a high $T (T = 2.0)$. The line shows a rapid collapse of the random coil chain into a region consisting of many interconverting semi-compact random states; the ground state is no longer stable so the chain spends most of its time in the entropically favoured denatured states. In contrast, at $T = 1.0$, the native state occurs for $\sim 40\%$ of the time while still being kinetically accessible.

for small proteins (Fig. 4d). However, the mechanism breaks down for long chains because the folding time increases exponentially with chain length owing to the fact that the number of semi-compact states increases faster than the number of the transition states. Thus, a modification of the present mechanism is required for larger proteins.

It is possible that proteins existing early in evolution were small enough to fold according to the three-stage random-search mechanism¹³. Because the pre-biotic and early biotic environment was hot, unusually thermostable proteins were required, such as those found in the most primitive bacteria that live at temperatures as high as 105°C ¹⁴. If so, the stability condition required a native state that corresponded to a deep energy mini-

mum for which the folding problem was solved simultaneously according to the present mechanism. As evolution progressed, longer proteins evolved. These proteins had to fold on the same timescale. One way of achieving this is by evolving proteins with sequences that have a larger difference between the native and non-native contact energies than the random folding sequences of the present model. Such sequences would have an even more pronounced global minimum in their potential surfaces, in line with the 'consistency principle'¹⁵ and the 'principle of minimal frustration'¹⁶. It is also in accord with the existence of a nucleus for folding⁶, or the early appearance of secondary structural elements^{7,17}, neither of which are found in the present model. As a result, the collapse would not result in random semi-compact

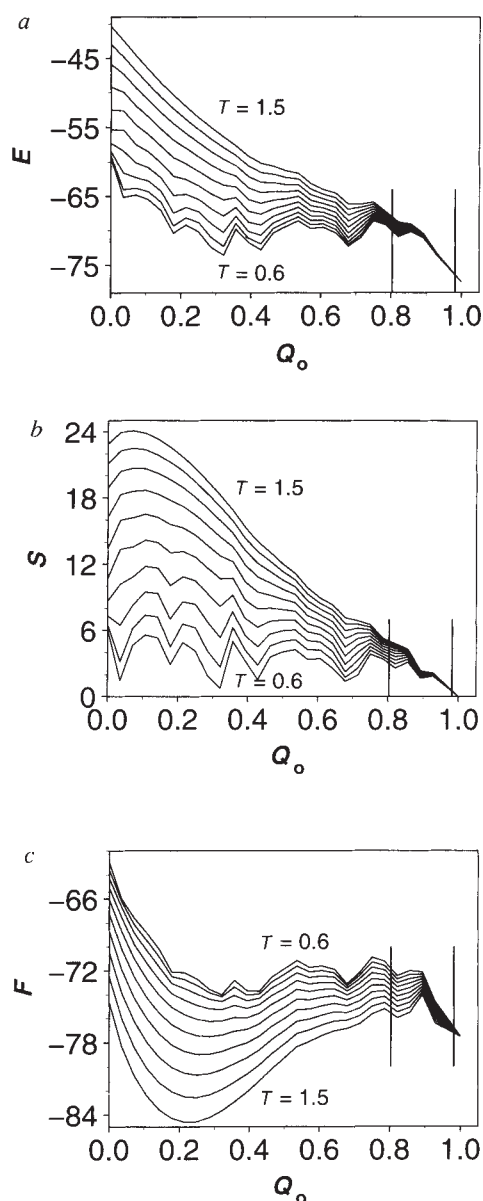
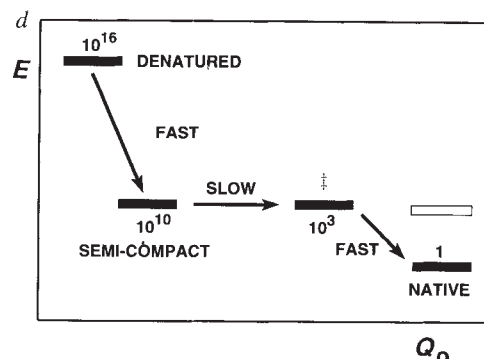


FIG. 4 Reaction profiles as a function of the reaction coordinate for the folding random sequence at $0.6 \leq T \leq 1.5$. *a*, Energy, *E*; *b*, entropy, *S*; *c*, free energy, *F*. The transition state lies between the two vertical lines at $0.83 \leq Q_0 \leq 0.96$. Profiles are calculated in temperature intervals of 0.1, using the partition function $Z(Q_0, T) = \sum_c \omega(Q_0, \epsilon) \exp(-\epsilon/(k_B T))$. *d*, Three-stage mechanism of folding for the present model (see text). The numbers of the states were obtained from Fig. 3a. The relative rates are obtained from 100 folding simulations (Fig. 1b). The empty rectangle indicates the energy of the native state of a non-folding sequence; in the non-folding sequence, the ground state would not be populated at a temperature sufficiently high to avoid being trapped as in Fig. 3c. The other states of a non-folding sequence appear at essentially the same positions as the corresponding states of a folding sequence. It can be estimated that a real protein of 80 residues has $2.57^{79} \approx 10^{32}$ possible main-chain conformations of which $1.7^{79} \approx 10^{18}$ are semi-compact¹². The number of the transition states can be extrapolated from a 27-mer as follows. In a 27-mer, 10^3 out of the 10^{10} semi-compact states are transition states; thus, in an 80-residue protein, about 10^6 out of the 10^{18} semi-compact states are likely to be transition states. According to a molecular dynamics simulation of native hydrated myoglobin at 300 K²², there are 52 main chain dihedral angle changes per 153 residues per 100 ps or 3 transitions per residue in 1 ns; conformational transitions in the semi-compact state are likely to be faster. Use of these numbers for an 80 residue protein that folds by the three-stage mechanism yields a rate-determining step of 10^{12} transitions which would correspond to a folding time to a molten globule state of about 3 s. This is close to the timescale observed in real proteins.



globules and the very favourable native contacts would lead more directly to a native-like molten globule state; the folding pathway in Fig. 3a would approximate a straight line from the random-coil to the 'native state'. Such a mechanism has been observed in folding simulations of long chains with highly stable native states¹⁸ (V. Abkevich, A. Gutin and E.S., unpublished results; A. Dinner, A.S. and M.K., unpublished results). Actual proteins could use an intermediate mechanism that might vary with the external conditions.

The results of this study may have implications for the prediction of the structure of a protein from its amino-acid sequence. The success of the three-stage random search in finding the pronounced global minimum on a potential surface suggests, at least for small proteins, that the bottleneck in structure prediction may be the derivation of a suitable potential function rather than the design of folding algorithms. □

1. Creighton, T. E. (ed.) *Protein Folding* (Freeman, New York, 1992).
2. Levinthal, C. in *Mossbauer Spectroscopy in Biological Systems* (eds Debrunner, P., Tsibris, J. C. M. & Münck, E.) 22–24 (Univ. Illinois Press, Urbana, 1969).
3. Shakhnovich, E., Farztdinov, G., Gutin, A. M. & Karplus, M. *Phys. Rev. Lett.* **67**, 1665–1668 (1991).
4. Šali, A., Shakhnovich, E. I. & Karplus, M. *J. molec. Biol.* **235**, 1614–1636 (1994).
5. Harding, M., Williams, D. & Woolfson, D. *Biochemistry* **30**, 3120–3128 (1991).
6. Wetlaufer, D. B. *Proc. natn. Acad. Sci. U.S.A.* **70**, 697–701 (1973).
7. Kim, P. & Baldwin, R. A. *Rev. Biochem.* **59**, 631–660 (1990).
8. Gö, N. & Abe, H. *Biopolymers* **20**, 1013–1031 (1981).
9. Honeycutt, J. D. & Thirumalai, D. *Biopolymers* **32**, 695–709 (1992).
10. Bryngelson, J. D. & Wolynes, P. G. *J. phys. Chem.* **93**, 6902–6915 (1989).
11. Shakhnovich, E. I. & Gutin, A. M. *Biophys. Chem.* **34**, 187–199 (1989).
12. Dill, K. A. *Biochemistry* **24**, 1501–1509 (1985).
13. Di Iorio, E. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **90**, 2025–2029 (1993).
14. Stetter, K. O. in *Frontiers of Life* (eds Trần Thanh Vân, J. K., Mounolou, J. C., Schnieder, J. & McKay, C.) 195–212 (Editions Frontières, Gif-sur-Yvette, France, 1992).
15. Gö, N. & Abe, H. *Adv. Biophys.* **18**, 149–164 (1984).
16. Bryngelson, J. D. & Wolynes, P. G. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7524–7528 (1987).
17. Karplus, M. & Weaver, D. L. *Nature* **260**, 404–406 (1976).
18. Taketomi, H. & Gö, N. *Int. J. Peptide Prot. Res.* **7**, 445–449 (1975).
19. Miyazawa, S. & Jernigan, R. L. *Macromolecules* **18**, 534–552 (1985).
20. Metropolis, N., Rosenbluth, A. W., Rosenbluth, M. N., Teller, A. H. & Teller, E. *J. chem. Phys.* **21**, 1098–1092 (1953).
21. Sykes, M. F. *J. chem. Phys.* **39**, 410–412 (1963).
22. Loncharich, R. J. & Brooks, B. R. *J. molec. Biol.* **215**, 439–455 (1990).

ACKNOWLEDGEMENTS. We thank A. Dinner, D. Šali, O. Ptitsyn, A. Gutin, G. Archontis, A. Cafisch, O. Becker and L. Demetrius for discussions concerning the protein folding problem. A.S. is a Fellow of The Jane Coffin Childs Memorial Fund for Medical Research. This investigation was aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research (A.S.), by David and Lucille Packard Fellowship (E.S.), and by a grant from the National Science Foundation and a gift from Molecular Simulations Inc. (M.K.). The computations were done on IBM RS/6000, Silicon Graphics Iris 4D, SUN Sparcstation, DEC Decstation, DEC Alphastation, and NeXT workstations.

Received 6 January; accepted 24 March 1994.

1. Creighton, T. E. (ed.) *Protein Folding* (Freeman, New York, 1992).
2. Levinthal, C. in *Mossbauer Spectroscopy in Biological Systems* (eds Debrunner, P., Tsibris, J. C. M. & Münck, E.) 22–24 (Univ. Illinois Press, Urbana, 1969).
3. Shakhnovich, E., Farztdinov, G., Gutin, A. M. & Karplus, M. *Phys. Rev. Lett.* **67**, 1665–1668 (1991).

Effects of activation-defective TBP mutations on transcription initiation in yeast

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TRANSCRIPTION initiation by RNA polymerase II is effected by an ordered series of general factor interactions with core promoter elements (leading to basal activity) and further regulated by gene-specific factors acting from distal elements¹. Both the general factor TFIID (refs 2, 3), including the constituent TBP (TATA-binding polypeptide)⁴⁻⁷ and associated factors⁸, and the interacting factor TFIIB (refs 9-11) have been implicated as targets for various activators. Towards an understanding of the basis for activator function, including the multiplicity of TBP interactions, we have now identified mutations in yeast TBP that selectively block activator (GAL4-VP16)-dependent but not basal transcription. We further show an effect of GAL4-VP16 on TFIIB recruitment to early preinitiation complexes, and that recruitment is disrupted by TBP mutations that impair its interactions with VP16 (L114K), TFIIB (L189K) or an unidentified component (K211L). Thus, GAL4-VP16 function seems to involve both direct interactions with TBP and a corresponding induction (or stabilization) of an activation-specific TBP-TFIIB-promoter complex.

For functional studies we used a reconstituted yeast system¹² in which basal transcription from reporter templates (Fig. 1a) was dependent on added TBP and activated in a manner dependent on the VP16 activation domain (fused to the GAL4 DNA-binding domain) and the presence of GAL4 binding sites in the reporter (Fig. 1b). A series of point mutants in the conserved carboxy-terminal core of yeast TBP was used to define residues important specifically for GAL4-VP16-dependent transcription. Because lysine and leucine residues are distributed extensively in this region, we focused on point mutants containing either lysine to leucine or leucine to lysine changes¹³. In assays with the basal activity normalized, all but three of 31 mutant TBPs showed either undetectable activities or both basal and activator-dependent activities comparable to those displayed by wild-type TBP (Fig. 2). In contrast, mutants L114K, L189K and K211L were defective for the response to GAL4-VP16, but nonetheless showed normal basal transcription by RNA polymerase II. In a further analysis of their specificity, all three mutants restored transcription from both pol I (35S ribosomal RNA)- and pol III (5S rRNA)-transcribed promoters as effectively as did wild-type TBP (Fig. 3a). Thus L114K, L189K and K211L are specifically defective in acidic activator-dependent transcription by RNA polymerase II.

To explore the molecular basis of the activation defects, interactions between mutant TBPs and the VP16 activation domain were investigated by coimmunoprecipitation. Consistent with previous results⁴, radiolabelled TBP bound to Protein A-VP16, but not to Protein A (Fig. 3b, lanes 1-6; I, P and S lanes reflect input, pellet and supernatant, respectively). Under these conditions, mutant L114K showed a severe loss of VP16 binding ability compared with wild-type TBP (lanes 7-9), whereas mutants L189K and K211L retained most of their ability to interact

with VP16 (lanes 10-15). In fact, control mutants (such as K97L and K239L) which effected normal basal and activated transcription showed comparably small losses of VP16 binding activity (lanes 16-30 and data not shown; see also figure legend), possibly reflecting (for all mutants) a minor (intrinsic) population of conformationally inactive forms of TBP that cannot bind VP16. These results show that one of the activation-defective mutations (L114K) affects direct interactions with the acidic activation domain.

Apart from their ability to bind TBP directly, acidic activators have also been reported to regulate interactions of TFIIA and TFIIB with TBP or TFIID^{9-11,14}. We therefore checked the abilities of the mutants to interact with TFIIA and TFIIB by band-shift analysis (Fig. 3c). Consistent with its inability to bind VP16, mutant L114K formed TBP-TFIIA, TBP-TFIIB and TBP-TFIIA-TFIIB promoter complexes as efficiently as did wild-type TBP. In contrast, and while normal with respect to TBP-TFIIA complex formation (lane 11), mutant L189K was deficient in forming a TBP-TFIIB complex stable to electrophoresis (lane 12) and thus has a reduced intrinsic DNA-binding potential. However, mutant L189K was still active in TBP-TFIIA-TFIIB complex formation (lane 13), indicating that stabilizing interactions dependent on TFIIA can compensate at least partially (to a level sufficient for basal transcription) for this reduced TFIIB binding potential. Similarly, because mutants L114K and K211L are both defective for independent DNA binding (observed under different ionic conditions) but not for basal transcription, one or more basal factors (such as TFIIA, TFIIB or downstream factors) must be able to compensate for this loss of intrinsic DNA-binding potential (as also observed for other TBP mutants¹⁵).

Taken together, these results (summarized in Fig. 3e) suggested that the altered TFIIB interaction potential of mutant L189K might reflect a defect that is manifested functionally only in activator-dependent (and not basal) transcription. To test this possibility, we did factor recruitment experiments with DNA templates fixed to agarose beads⁹⁻¹¹. The immobilized CYC1 promoter was incubated with TBP and TFIIB in the presence or absence of GAL4-VP16. After washing, complexes were assayed for TBP and TFIIB levels by immunoblot and for transcription potential by complementation with TBP- and TFIIB-deficient yeast extracts containing other essential factors. In the absence of GAL4-VP16, wild-type TBP and mutants L114K and L189K resulted in comparable levels of bound TBP and TFIIB, as well as comparable levels of basal transcription (Fig. 3d). Incubation with GAL4-VP16 noticeably increased both the overall level of transcription and the amount of TFIIB recruited into promoter complexes in the presence of wild-type TBP, thus indicating an activation mechanism involving enhanced TFIIB recruitment (or stabilization) and consistent with results in mammalian systems⁹⁻¹¹. However, activation-defective mutants L114K and L189K both showed marked reductions in the levels of activated transcription and of recruited TFIIB under the influence of the activator (Fig. 3d, lanes 3-6). Therefore, consistent with results of band-shift analyses showing a quantitative loss of intrinsic TFIIB binding potential in the L189K mutant (Fig. 3c), the recruitment assay also indicates that the L189K mutation affects direct TBP-TFIIB interactions but, importantly, just those that are somehow important only for activator-enhanced transcription. Furthermore, the results of the L114K analyses (inability to bind VP16 or to mediate enhanced TFIIB recruitment) suggest that VP16 can contribute to enhanced TFIIB recruitment through interactions with TBP. This does not exclude the possibility that enhanced TFIIB recruitment might also involve TFIIB-VP16 interactions⁹⁻¹¹.

Figure 4 illustrates the mutated positions in the three-dimensional structure of TBP¹⁶. The loop region (between S2' and S3') containing L189 is particularly interesting because of its conformational flexibility and potential, as a result of its exposed position, for direct protein-protein interactions¹⁶. The

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a

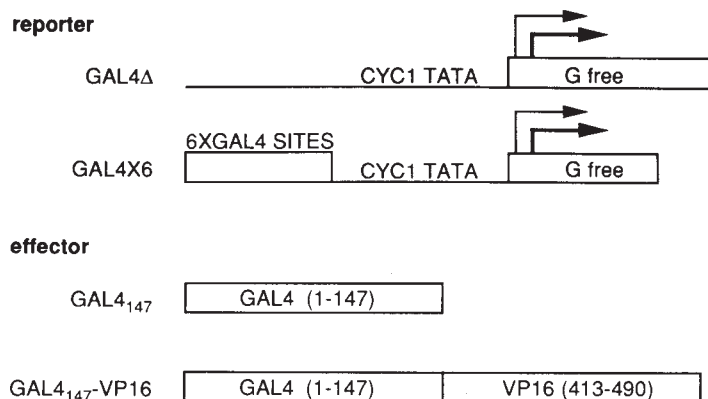


FIG. 1 TBP-dependent transcription system. *a*, Constructions used in this study. The template DNAs for *in vitro* transcription contain a TATA box from the CYC1 promoter with or without six GAL4 binding sites. The basal template contains a G-less cassette of 400 base pairs and the activator-responsive template with GAL4 binding sites has a 100-base-pair-truncated G-less cassette^{25,26}. The chimaeric GAL4-VP16 protein contains the amino-terminal 147 amino acids of GAL4 (with the DNA-binding domain) fused to the C-terminal 78 amino acids of VP16. *b*, *In vitro* transcription analysis. Templates with and without GAL4 DNA-binding sites were included in each reaction to ascertain that activation was mediated through sequence-specific DNA binding. Transcription reactions were done in the reconstituted yeast system in the presence of no activator (lane 1), GAL4-VP16 fusion protein (lanes 2 and 3), truncated GAL4 (residues 1–147) (lanes 4 and 5) or no bacterially expressed yeast TBP (lanes 3 and 5). Specific transcripts from control (GΔ) and activator-responsive (G6X) templates are indicated by arrows. METHODS. The *in vitro* transcription system used in this study was reconstituted as described before¹². GAL4-VP16 was expressed in and purified in bacteria²⁷.

b

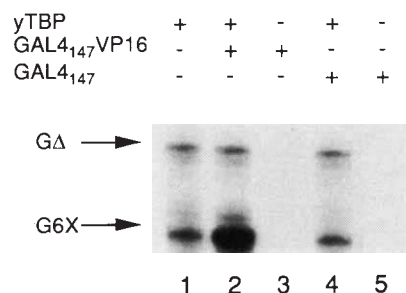
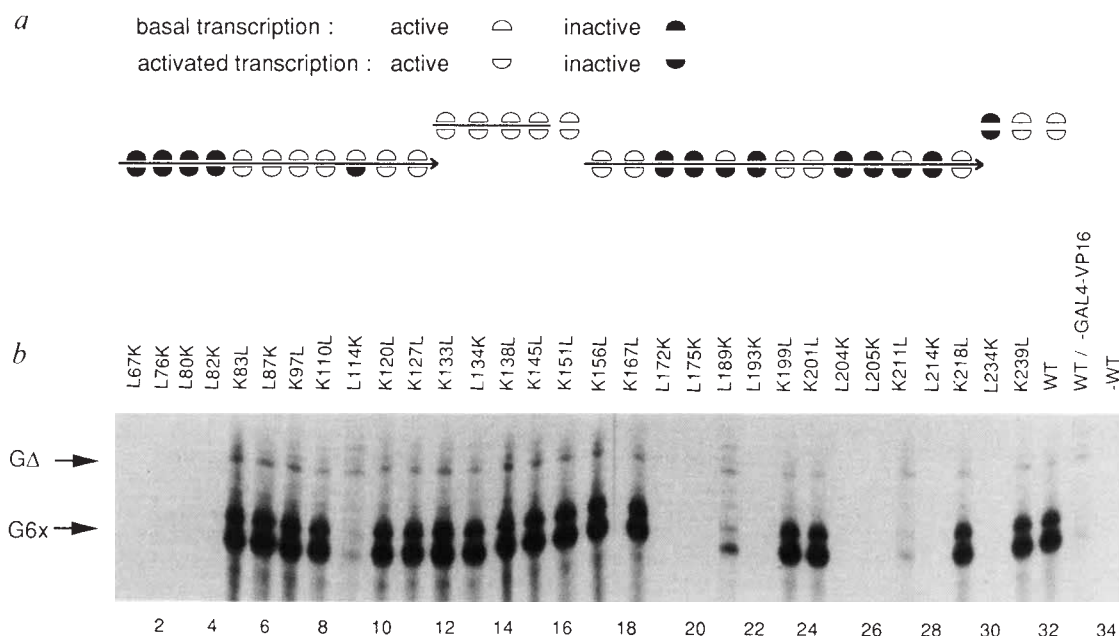


FIG. 2 Analysis of yeast TBP point mutants for activator-dependent versus basal transcription. *a*, Schematic representation of structural motifs in TBP and summary of the effects of mutations on basal transcription and activation by GAL4-VP16. The C-terminal conserved core domain of TBP contains two direct repeat domains (arrows) separated by a region rich in basic amino acids (solid line). The unfilled and filled half circles indicate the basal versus activator-dependent transcription activities of the mutant TBPs indicated above the corresponding lanes in *b*. *b*, Transcription analysis with TBP mutants. Lanes 1–31 show analyses of responses to GAL4-VP16 by TBP species mutated at the positions indicated above each lane (L67K indicates leucine changed to lysine at position 67 and so on). Lanes 33 and 34 contain, respectively, wild-type TBP without GAL4-VP16 and GAL4-VP16 without TBP. The arrows indicate specifically initiated transcripts from basal (GΔ) and activator-responsive (G6X)^{25,26} templates.



templates. METHODS. Wild-type and mutant TBPs were expressed as hexahistidine fusion proteins in bacteria and purified by nickel column chromatography to about 80% purity²⁸. Each purified protein was assayed in the TBP-dependent reconstituted system as described in Fig. 1.

other two mutations (L114K and K211L) map to strands (S4 and S5', respectively) in the concave DNA-binding surface¹⁷. This raises the possibility that TBP has overlapping or interdigitated DNA binding and activation-specific domains¹⁸⁻²⁰, whose positions might change during preinitiation complex assembly²¹. Related to this possibility, concomitant or subsequent conformational changes in TBP induced by TFIIA¹⁵ or by other factors might allow activator-specific interactions of TBP. At the same

time, activation defects might result from subtle conformational changes in mutant TBP complexes.

The importance of the activation-specific TBP-TFIIB interactions demonstrated here is supported by previous data⁹⁻¹¹ indicating activator-mediated recruitment of human TFIIB to (or subsequent stabilization within) a TBP or TFIID promoter complex and supports a recent speculative model²² invoking qualitative activator-dependent changes in TBP-TFIIB inter-

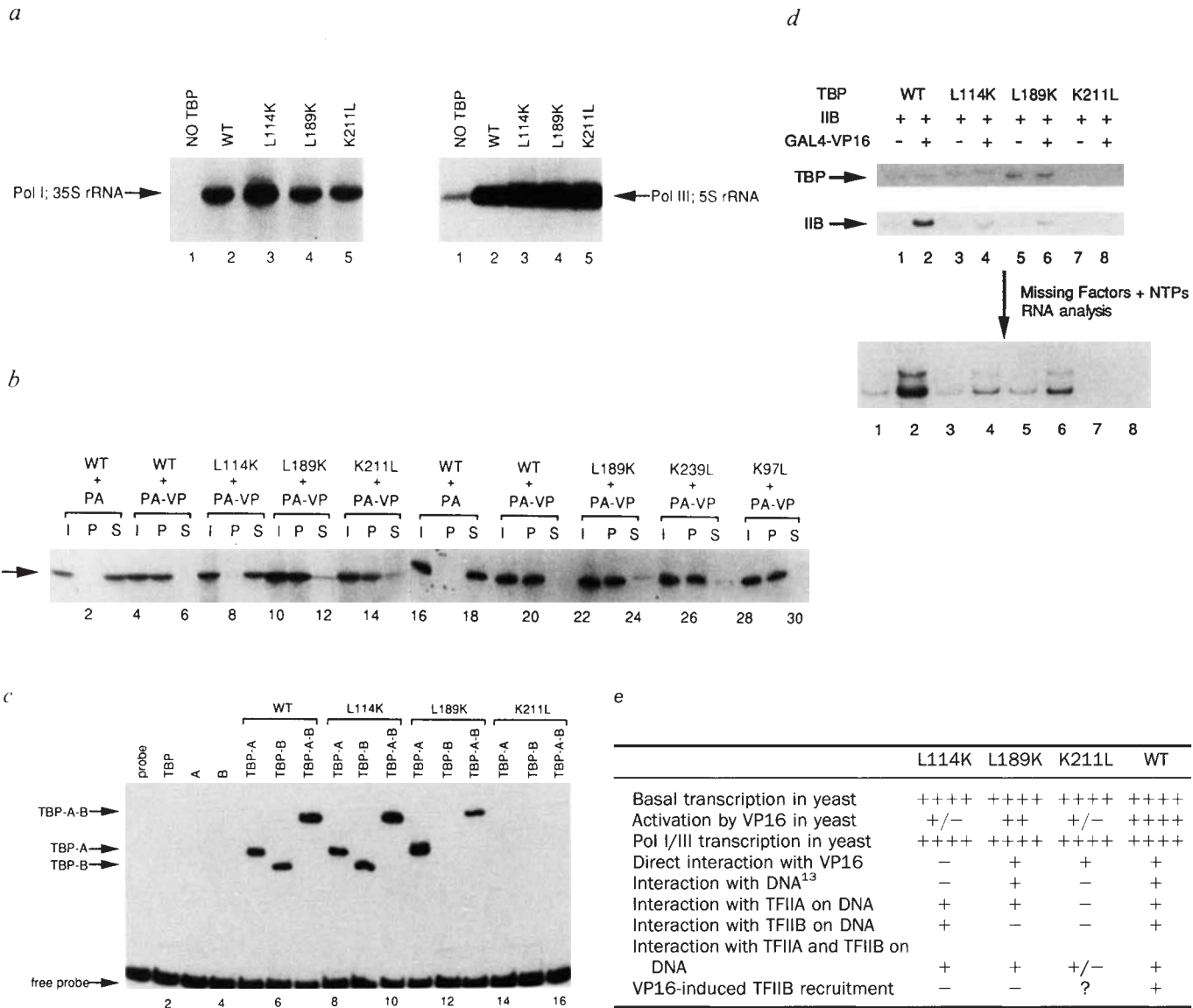
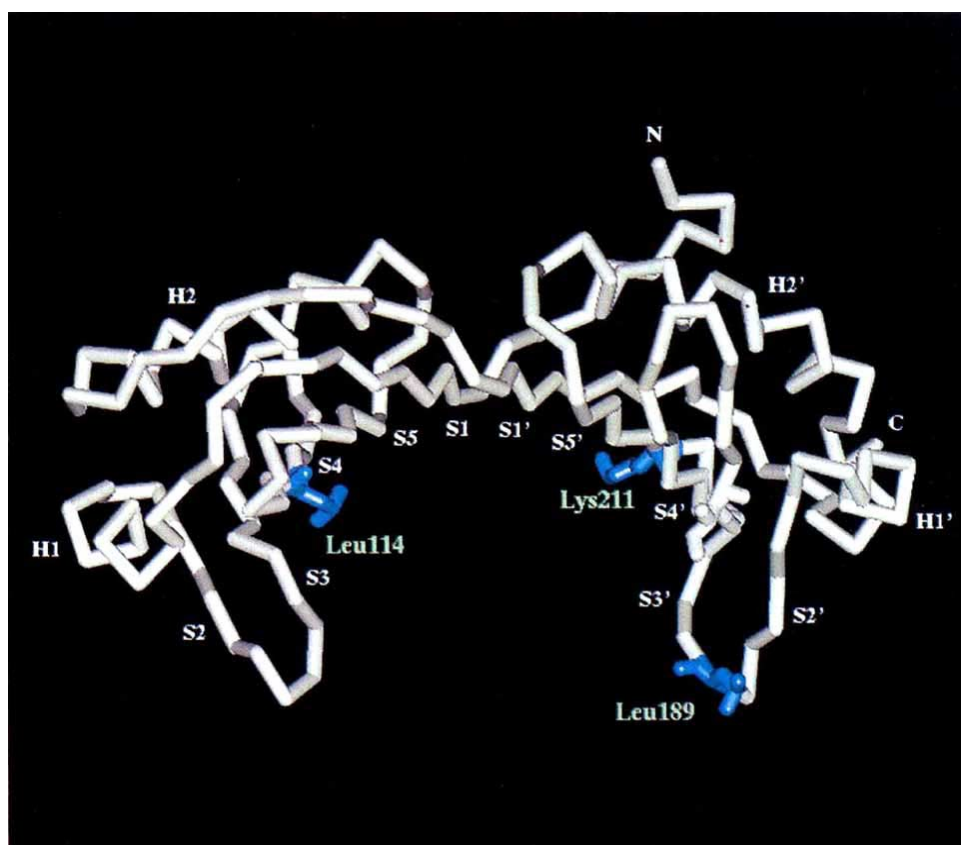


FIG. 3 Characterization of TBP mutants defective in activation but not basal transcription. **a**, Pol I and III transcription activity. **b**, Interactions with the acidic activation domain of VP16. **c**, Interactions with TFIIA and TFIIB. **d**, TBP-TFIIB promoter complex formation on immobilized templates. **e**, Summary of properties of activation-defective TBP mutants.

METHODS. **a**, Extracts derived from a temperature-sensitive yeast strain containing the I143N TBP mutant were used to test the ability of activation-defective mutants to rescue pol I and III transcription²⁸, because TBP-dependent transcription system in Figs 1 and 2 was found not to support pol I and III transcription well. **b**, The Protein A-VP16 fusion protein was purified as described (Pharmacia). ³⁵S-methionine-labelled TBPs were generated by *in vitro* translation of corresponding complementary DNA-derived messenger RNAs. *In vitro* labelled proteins were incubated with Protein A or Protein A-VP16 in buffer D (10% glycerol, 100 mM KCl, 20 mM Tris-Cl, pH 7.9, 0.2 mM EDTA, 0.03% NP-40, 1 mM

DTT and 20 µg ml⁻¹ BSA) and then coimmunoprecipitated with IgG-Sepharose beads^{4,11}. Bound proteins present in the pellet (P) were separated, along with input proteins (I) and unbound proteins in the supernatant (S), on a 15% acrylamide gel. Except for mutant L114K, all the mutants analysed for transcription in Fig. 2 showed normal VP16 binding (data not shown). **c**, Band-shift analyses with a TATA-containing oligonucleotide were as described²⁹. Under these conditions, stable TBP binding requires the presence of TFIIA or TFIIB. Yeast TFIIA³⁰ (TOA1 and TOA2) and TFIIB were purified from bacteria. **d**, Immobilized template *in vitro*-factor recruitment and transcription assays were as described⁹⁻¹¹. Complementing fractions with essential factors were prepared by immunodepletion (with anti-yTFIIB antisera) of yeast TFIIB from fractions described in Fig. 1. The L189K TBP-TFIIB promoter complex is obviously more stable in this assay than in the band-shift assay, presumably because of more stringent conditions in the latter case.

FIG. 4 Schematic figure showing the positions of TBP mutations that selectively affect activation by GAL4-VP16. The X-ray crystallographic analysis of the *Arabidopsis* homologue of yeast TBP has shown that the conserved core structure is comprised of two highly symmetric domains, each with two α -helices and an α -stranded anti-parallel β -sheet¹⁶. The α -carbon backbone is shown as a solid white line. Also shown are side chains of the three activation-defective mutants, L114, L189 and K211, with possible interaction sites indicated in blue.



actions. Our data suggest that GAL4-VP16 may either recognize a TBP-DNA complex and induce an activation-specific conformation that aids functional interactions (and increased recruitment) of TFIIB or, alternatively, that it may recognize and stabilize a conformation-specific TBP-TFIIB-DNA complex,

and, consequently, enhance TFIIB recruitment. By analogy to the situation in mammalian cells^{9,11}, the enhanced TFIIB recruitment mediated by GAL4-VP16 in conjunction with TBP may nonetheless require additional cofactors^{12,23,24} for the functional consequences (enhanced transcription) to be realized. □

Received 7 February; accepted 3 March 1994.

1. Roeder, R. G. *Trends biochem. Sci.* **16**, 402-427 (1991).
2. Horikoshi, M., Hai, T., Lin, Y.-S., Green, M. R. & Roeder, R. G. *Cell* **54**, 1033-1042 (1988).
3. Workman, J. L., Abmayr, S. M., Cromlish, W. A. & Roeder, R. G. *Cell* **55**, 211-219 (1988).
4. Ingles, C. J., Shales, M., Cress, W. D., Triezenberg, S. J. & Greenblatt, J. *Nature* **351**, 588-590 (1991).
5. Lee, W. S., Kao, C. C., Bryant, G. O., Liu, X. & Berk, A. J. *Cell* **67**, 365-376 (1991).
6. Horikoshi, N. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 5124-5128 (1991).
7. Lieberman, P. M. & Beck, A. J. *Genes Dev.* **5**, 2441-2454 (1991).
8. Goodrich, J. A., Hoey, T., Thut, C. J., Admon, A. & Tjian, R. *Cell* **75**, 519-530 (1993).
9. Lin, Y.-S. & Green, M. R. *Cell* **64**, 971-991 (1991).
10. Choy, B. & Green, M. R. *Nature* **366**, 531-536 (1993).
11. Kim, T. K. & Roeder, R. G. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
12. Fianagan, P. M., Tschochner, H., Kelleher, R. J. III, Sayre, M. H. & Kornberg, R. D. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7659-7663 (1992).
13. Yamamoto, T. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 2844-2848 (1992).
14. Meisterernst, M. & Roeder, R. G. *Cell* **67**, 557-567 (1991).
15. Lee, D. K., DeJong, J., Hashimoto, S., Horikoshi, M. & Roeder, R. G. *Molec. cell. Biol.* **12**, 5189-5196 (1992).
16. Nikolov, D. B. et al. *Nature* **360**, 40-46 (1992).
17. Kim, Y., Geiger, J. H., Hahn, S. & Sigler, P. B. *Nature* **365**, 512-520 (1993).
18. Keaveney, M., Berkenstam, A., Feigenbutz, M., Vriend, G. & Stunnenberg, H. G. *Nature* **365**, 562-566 (1993).

19. Ptashne, M. *A Genetic Switch, Gene Control and Phage 1* (Cell Press and Blackwell Scientific, Cambridge, 1986).
20. Schena, M., Freedman, L. P. & Yamamoto, K. R. *Genes Dev.* **3**, 1590-1601 (1989).
21. Kassavetis, G. A. et al. *Cell* **71**, 1055-1064 (1992).
22. Hahn, S. *Nature* **363**, 672-673 (1993).
23. Fianagan, P. M., Kelleher, R. J. III, Sayre, M. H., Tschochner, H. & Kornberg, R. D. *Nature* **350**, 436-438 (1991).
24. Berger, S. L. et al. *Cell* **70**, 251-265 (1992).
25. Kim, T. K. & Roeder, R. G. *J. biol. Chem.* **268**, 20866-20869 (1993).
26. Kim, T. K. & Roeder, R. G. *Nucleic Acids Res.* **22**, 251 (1993).
27. Chasman, D. I., Leatherwood, M. C., Carey, M., Ptashne, M. & Kornberg, R. D. *Molec. cell. Biol.* **9**, 4746-4749 (1989).
28. Kim, T. K. & Roeder, R. G. *J. biol. Chem.* **269**, 4891-4894 (1993).
29. Auble, D. T. & Hahn, S. *Genes Dev.* **7**, 844-856 (1993).
30. Ranish, J. A., Lane, W. S. & Hahn, S. *Science* **255**, 1127-1129 (1992).

ACKNOWLEDGEMENTS. We thank D. B. Nikolov and S. K. Burley for the figure showing the structure of TBP and Y. J. Kim for anti-yeast TFIIB antiserum. R.J.K. was supported by the Medical Scientist Training Program from the NIH. This study was supported by NIH grants to R.D.K., M.H. and R.G.R., by a Human Frontier Science Program grant to R.G.R., and by general support from the Pew Trusts to The Rockefeller University.

Small-world solutions

Highlighted this week — a handy device for replica plating microbial colonies on solid growth media, a kit for the direct colorimetric detection of hepatitis B DNA and a nonradioactive reverse transcriptase assay for the detection of HIV-1.

The new **mycoplasma removal agent** available from ICN Biomedicals is designed to kill mycoplasma rather than inhibit its growth (*Reader Service No. 100*). This



MRA keeps cultures mycoplasma-free.

water-soluble oxo-quinoline-carboxylic acid derivative is said to be effective against both cultured and 'wild-type' mycoplasma. Each shipment is supplied with a protocol.

Schleicher & Schuell's Accutran **replica plater**, designed to fit 100-mm Petri dishes, is a new device for replica plating microbial colonies on solid growth media (*Reader Service No. 101*). This disposable, sterilized unit can be used to create up to 12 colony transfers from a single master plate. It consists of a moulded, nylon velvet that has a surface akin to velvetene cloth. Replicas are made by lowering the plates onto the replica plater. Alignment bumpers are designed to guide the Petri dishes into place and



Replica plating gadget.

256

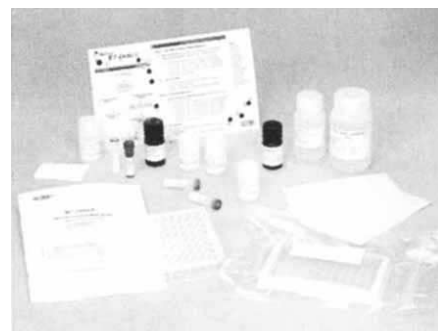
prevent the smearing of colonies. Packs of 25, 100 or 500 are available.

■ Detection

The method used in the new enhanced microplate hybridization assay for the direct colorimetric **detection of hepatitis B virus DNA**, available from Enzo Diagnostics, is based on a hybridization and detection procedure performed in a 96-well microtitre plate or well strip (*Reader Service No. 102*). Serum or plasma, processed by two 5-min digestion steps, is assayed directly by hybridization of HBV DNA to a microwell-bound capture probe, followed by binding a biotinylated signalling probe to the captured target. The immobilized target HBV DNA is then visualized with a biotin-binding, signal-generating complex of streptavidin and horseradish peroxidase. Sensitivity has been maximized by the addition of an anti-biotin antibody and biotinylated secondary antibody. A positive reaction is indicated by generation of colour which can be measured in a microplate reader and quantified for copy number by using HBV DNA titration standards. The kit contains probe reagents, probe-coated microplate strips for 48 duplicate assays, detection reagent, substrate, chromogen, wash buffers and positive controls. Titration standards are provided in a companion kit.

The MicroSilver kit from Polysciences can be used for the **detection of fungi and *Pneumocystis carinii*** (*Reader Service No. 103*). The ammoniacal silver staining procedure used in the kit is designed to replace methenamine silver methods. The kit offers a choice of either periodic acid or chromic acid as oxidizing agents to suit specific types of specimens. It features a microwave protocol. All reagents are provided in working concentrations: the ammoniacal silver solution is prepared just prior to use in the calibrated bottle provided. Sufficient reagents are provided for staining 500 specimen slides. Positive controls for *Candida albicans* and *P. carinii* are available separately.

Du Pont's RT Detect nonradioactive reverse transcriptase **assay for the detection of HIV-1** is said to require less than an hour of preparation time, with results available in four (*Reader Service No. 104*). The v-well microplate included in the kit allows for high-throughput testing. The kit's lysis buffer provides for resuspension of viral pellets and is said to stabilize samples for storage at or below -20°C for at least a month. Kits

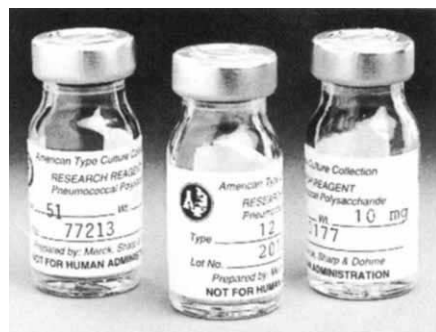


RT Detect kit for the detection of HIV-1.

may be shipped and stored refrigerated, eliminating the need to freeze and thaw before use. Du Pont says that two recent studies in the July '93 and February '94 issues of the *Journal of Antimicrobial Agents and Chemotherapy* indicate that the assay can be used to demonstrate *in vitro* RT drug resistance.

■ Reagents

A range of **pneumococcal and meningococcal purified polysaccharides** can now be obtained from the American Type Culture Collection (*Reader Service No. 105*). The line-up includes 25 types of purified



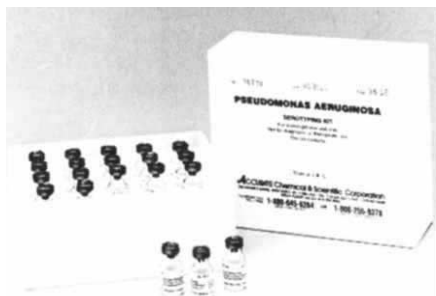
Pneumococcal and meningococcal polysaccharides — new from the ATCC.

pneumococcal polysaccharides, including the components of the 23-valent pneumococcal polysaccharide vaccine against *Streptococcus pneumoniae*, and one type of meningococcal polysaccharide, W-135. In addition to their use in assaying for type-specific antibody, these antigens may be useful in the analysis of other immune responses and immunodeficiency studies.

Two new **antibodies**, one against *Cryptococcus neoformans*, the other against *Toxoplasma gondii*, are now available from Dako for use on routinely processed tissue (*Reader Service No. 106*). The first reacts with cryptococcus capsular polysaccharide

present in *C. neoformans* var. *neoformans* (serotypes A and D) and *C. neoformans* var. *gatti* (serotypes B and C). It is said to cross-react with *C. albidus* and *Torulopsis galbrata* but not *Candida albicans*. *C. neoformans*, an encapsulated yeast, is the causative agent of *Cryptococcosis*, which can disseminate to produce a fatal form of meningitis. Dako's second antibody labels both tachyzoites and bradyzoites in infected tissue when using the immunoperoxidase technique. In indirect fluorescence assays, Dako says that it has been shown to stain the outer surface of tachyzoites of two strains of *T. gondii* (RH and T626). Tissue infected with the following organisms is said not to react with anti-*T. gondii*: *Cryptosporidia*, *Microsporidia*, *Histoplasma capsulatum*, *Candida*, *Blastomyces*, *Pneumocystis carinii*, *Entamoeba histolytica*, *C. neoformans* or *Mycobacterium tuberculosis*.

A monoclonal antibody-based **kit for serotyping *Pseudomonas aeruginosa*** is now available from Accurate Chemical &



Bacterial agglutination test for serotyping *Pseudomonas aeruginosa*.

Scientific (Reader Service No. 107). Monoclonal antibodies specific to each of the 17 O antigens of *P. aeruginosa* will induce agglutination of the homologous bacterial strain by forming a lattice that is visible as 'clumps': in the absence of homology, the bacteria will remain in a smooth suspension. Test results can be obtained within 2 min, says AC & S.

The new Biosyn range of active site-directed **protease affinity labels and inhibitors** are designed for the detection and characterization of active proteases isolated from biological specimens such as biopsy homogenates (normal and tumour), microbial extracts, parasitic fluke media, and cell-line conditioned media (Reader Service No. 108). Trypsin, calpain, cathepsins B and L, trypsin, chymotrypsin and related serine and cysteine proteases can be identified using the labels in conjunction with western blot analysis. Visualization is with alkaline phosphatase-streptavidin.

Over 20 preweighed **antibiotic supplements** covering a wide range of media can now be obtained from Difco Laboratories under the Betalab label (Reader Service No. 109). Included among these are supplements for isolating *Legionella* spp., *Helicobacter*



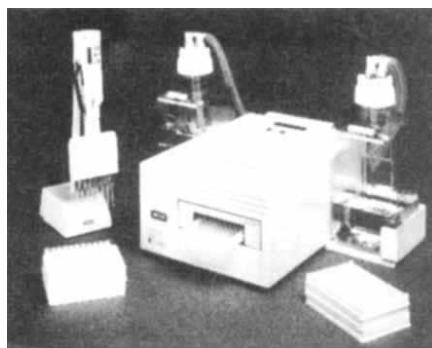
Selective antibiotic supplements.

pylori and *Clostridium difficile*. The company is also offering a 16-page **product guide for bacterial antigens and antisera** used for both diagnostic and epidemiological purposes. In all, 299 products are listed. The guide lists a range of common and rare antisera for serotyping *Salmonella*, *Shigella*, *Haemophilus influenza*, *Neisseria meningitidis*, *Pneumococcus*, *Pseudomonas aeruginosa*, *Streptococcus*, *Listeria*, *Escherichia coli*, *Bordetella* and *Candida*.

■ Instruments

The Bioscreen **automated microbiology analyser** from Labsystems, now available from the distributor Life Sciences International, is a computer-controlled robotic shaker, incubator, reader and analyser — all in one — designed for the automation of microbiological assays (Reader Service No. 110). Using 100-well microplates, the instrument can be set to run for up to 300 hours. It features a programmable incubation range of 1–60 °C. The accompanying software can be used to run protocols defined by the operator, record results and manipulate data.

ProWash is the new **microplate washer** from Biohit Oy intended for cell washing and ELISA work (Reader Service No. 111). Features of the strip washer include software control over 8- and 12-well protocols, so



ProWash plate washer.

there is no need for head switching, automatic voltage selection and autoprime, 20 storage protocols, vacuum control and programmable washing protocols. ProWash also determines the well depth and has nine head settings for optimizing the drying of plates.

Independent is the new **cordless, programmable pipetting device** from Integra Biosciences available in 8- and 12-channel versions and in three volume ranges — 5–250 µl, 15–850 µl and 15–1,250 µl (Reader Service No. 112). The device is programmed directly from its handle. An integrated display indicates the individual steps — filling, dispensing or mixing — being performed. The 8-channel version is also available as an 'expandable' version: by moving a knob on the handle, the spacing between pipette tips can be altered from the closed position (9.11-mm spacing) to the open position (14.5-mm spacing). This feature allows, for example, liquids to be transferred from microtitre plates to 48-well plates without having to resort to the use of single-channel pipettes.

■ Consumables

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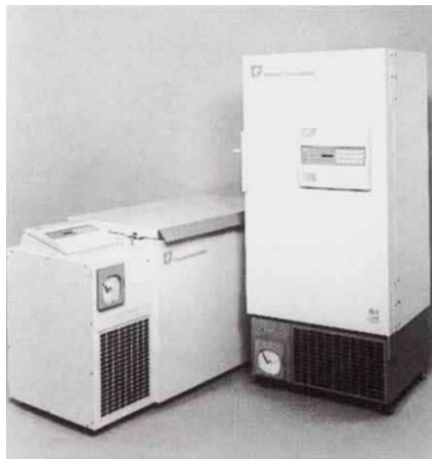
mobility and the production of infective virus in cell culture. With this in mind, Cellon Sàrl has introduced a range of IVSP **surface-activated PETG flasks** which provide a cell substrate distinct from that offered by polystyrene vessels (*Reader Service No. 113*). Suggested uses for the flasks include cultivation of cancer cell lines, drug testing, expression of cell-surface receptors, the conditioning of media, and AIDS research.

Costar now offers a range of certified **nonpyrogenic tissue culture flasks and clusters** (*Reader Service No. 114*). Every lot of Costar flasks and clusters now has a stated endotoxin level of less than $0.5 \text{ E}_u \text{ ml}^{-1}$ (by the U.S.P.XXII bacterial endotoxins test). This line is designed to complement the company's existing range of nonpyrogenic labware which includes serological pipettes and specialty tips for single-channel pipettors.

■ Incubators/freezers

Fisher Scientific has added two new additions to its Isotemp line of standard laboratory **incubators** (*Reader Service No. 115*). The Isotemp Standard Model features digital temperature selection in 0.1°C increments with a stated $\pm 0.1^\circ\text{C}$ sensitivity of control. A battery backup retains setpoint temperature values should power flow to the unit be interrupted. For light-duty laboratory assignments, such as microbiology culture growth studies, drying and staining procedures and coliform determinations, Fisher Scientific recommends its Isotemp Economy Model. Temperatures can be selected in 0.5°C increments and again the stated control sensitivity is $\pm 0.1^\circ\text{C}$. Both models are designed for the 30 to 75°C (86 to 167°F) temperature range and operate on gravity flow convection. Low-watt-density heaters are used; a backup heater is incorporated should the primary one fail. Three sizes are available (2.5, 3.75 and 5.0 cu. ft) to accommodate a variety of workloads, and all models are stackable. Both models also feature an electrical receptacle inside the chamber to permit connection of a stirrer, shaker or other apparatus.

Forma Scientific's Model 8500 Series of **ultra-low temperature freezers** use environmentally-safe refrigerants that are non-flammable, are CFC- and HCFC-free, and operate at temperatures down to -86°C



Environmentally-friendly freezers.

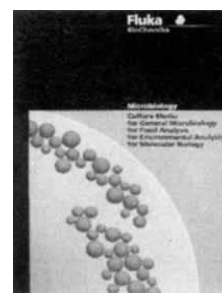
(*Reader Service No. 116*). Chest and upright models are available. The new Peak control system is an internal control system designed to protect compressors. Additional enhancements include a compressor that is 50 per cent larger for increased removal of heat, front-to-back airflow to enhance cooling of the compressors, tubeaxial fans designed to increase airflow through the compressor housing, and a reusable polyester foam filter. All freezers in the series have foamed-in-place urethane insulation and stainless steel interiors.

■ Catalogues/books/services

Now out — the 1994 **cryopreservation catalogue** from MVE Cryogenics (*Reader Service No. 117*). Featured products include the TEC 2000 and TEC 1000 microprocessor control systems, and the company's complete line of cryogenic storage equipment.

Fluka Chemical's 131-page **microbiology catalogue** is now available (*Reader Service No. 118*). Featured products include culture media for general microbiology,

environmental and food analysis, and molecular biology. Additionally, the catalogue features base ingredients, antibiotics, additives, dyes and indicators. Information on use of the products, with reference data, is included. Of interest is a product comparison chart, a worldwide list of culture collections, and a list of other microbiology publications available from Fluka.



Fluka's new products guide.

The *Oxoid Vade-Mecum of Microbiology* is a useful **aide-memoire for microbiologists** (*Reader Service No. 119*). Authored by microbiologist, Eric Bridson, the handbook is a dictionary of genera, alphabetically listing all the common, and medically important, micro-organisms under four main divisions — bacteriology, mycology, virology and parasitology. For each genus, major characteristics, species and recommended culture media for growth and isolation are given.

A broad array of **microbiological services** is being offered by the Belgian Coordinated Collections of Micro-organisms (BCCM) (*Reader Service No. 120*). Included among these are services, lists of which can be found in catalogues that can be obtained from BCCM, covering areas such as identification, molecular fingerprinting, safe deposit and contract research (isolation/screening of new strains). BCCM also serves as an international depository of filamentous and yeast fungi, bacteria, genetic material (plasmids, RNA and onco-genes), human and animal cell lines, genetically modified cell lines and hybridomas. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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Reader Service No. 11

Australian pharmaceutical industry observed

Jeremy Wurm

Multinationals are investing increasingly in local operations, with indigenous rather than expatriate managements. Scientific jobs are mainly filled by Australian graduates, many with overseas experience.

WHETHER Australia likes it or not, the country is competing in the global marketplace, and Australians have been forced to forsake a long-standing preoccupation with "the tyranny of distance". The fact is that Australia is now firmly entrenched as a Pacific Rim nation, and the pharmaceutical industry has adapted to enable it to prosper. Major changes in the regulatory and pricing regulation systems have increased the local industry's international competitiveness, and Australian scientific, manufacturing and clinical research standards are second to none.

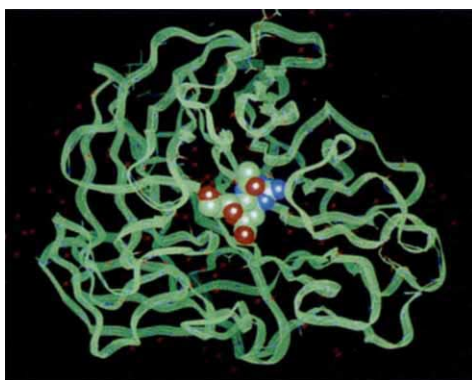
Every organization operating in Australia has to contend with major issues raised by the geographic and demographic characteristics of the continent itself. A highly urbanized country with only 17 million inhabitants, Australia is making progress in its transition into the international mainstream.

Sydney and Melbourne account for over half of the country's population. In terms of pharmaceutical activity, most organizations are located in Sydney, and to a lesser extent in Melbourne. However, Adelaide, Brisbane and Perth can all legitimately claim to be Head Office locations for a handful of pharmaceutical and related organizations.

Foreign ownership

Most Australian pharmaceutical companies are foreign owned, with a few notable exceptions. Off-shore decision making is further complicated in some instances by the fact that a number of Australian subsidiaries report to international headquarters through a regional office, in a city such as Singapore or Tokyo. Physical distance from the centre of international decision making can pose a real problem, especially when compounded with the modest commercial value of the Australian marketplace in relation to the United States, Japan and Europe.

A major disincentive to involvement by foreign companies in the Australian marketplace has always been the volume of the market itself (less than 2 per cent of the world market). Transport costs between Australia and other markets are significant, as are freight charges within the country itself. Transfer pricing repre-



Glaxo's sialidase inhibitor, now under development for the treatment of influenza, was designed by scientists at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the Victorian College of Pharmacy (Monash University). See Nature 363, 418-423 (1993).

sents another area where potential investors in Australia have often found obstacles to entering the local market. Relatively high total employment costs in Australia contribute further to the dilemma.

Counterbalancing all of these issues, however, is the government's Factor F Programme, which has been implemented with the aim of providing manufacturers with financial incentives to invest in local operations. At least until 1999, the pharmaceutical industry has been afforded the opportunity to overcome many of the

barriers to investment which have existed in the past.

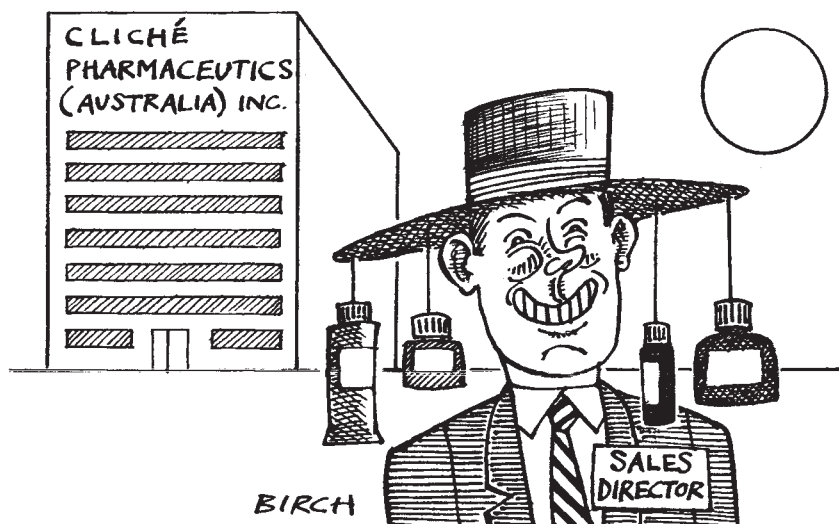
Harmonization

'Harmonization' has been a feature of the past few years, not only in the production context, but also in the broad areas of research and development, regulatory affairs and clinical medicine. In each of these fields, Australia can claim to have satisfied or exceeded the most stringent international performance criteria.

In recent years, the Australian industry has demonstrated a genuine commitment to self-regulation. This has had a range of positive benefits, which include an overall increase in product quality, together with an improvement in the standards applied to product promotion.

The drug evaluation process in Australia has been streamlined and is now compatible with the procedures applied in major world markets. The Therapeutic Goods Act of 1989 represented a major step in improving the relationship between industry and government. In 1991, an independent review was commissioned, and Professor Peter Baume assessed the drug evaluation system, identifying areas for further rationalization.

The Baume report concluded with a range of recommendations, all of which were accepted by the government. The implementation of these recommendations has seen Australian regulatory re-





Glaxo Australia's principal production site and headquarters at Boronia, Victoria.

quirements achieve consonance with European standards. Approval times have been reduced, administrative procedures simplified, and the whole process has become more effective.

The net result is that the relationship between government departments and the industry as a whole has improved considerably. These fundamental changes have encouraged foreign companies to launch new products in Australia, and facilitated the participation of local manufacturers in other world markets.

Manufacturing

Many foreign companies have taken the view that it is not cost effective to maintain full-scale local production facilities, despite the incentives offered by the Factor F pricing scheme. The result is that manufacturing in Australia is often confined to the less complex formulations, or the organization restricts itself to local packaging of imported finished product.

Until a decade ago, there had been a trend for pharmaceutical companies in Australia to wind down their production facilities. In the light of Factor F and other fundamental changes, there are now clear signs of reinvestment in pharmaceutical manufacturing. There is a concerted effort to achieve compliance with international standards of good manufacturing practice, encouraged by tax concessions which are available to pharmaceutical producers who have gained accreditation.

Australia is a signatory to the Pharmaceutical Inspection Convention. From a commercial point of view, an obvious advantage has been the facilitation of a genuine export market for the Australian industry, and the ability of Australian companies to import from overseas.

Historically, Australia has been a net importer of pharmaceutical specialities,

although there is now a real opportunity for companies to take advantage of the much larger potential markets for pharmaceuticals, in South-East Asia, and beyond. Exporting from Australia has grown dramatically, and the Pacific Rim represents a vast, and previously overlooked, source of income; better pricing has been a major supporting influence and improved regulatory procedures have also had a positive effect.

Scientific development

In almost every case, multinational pharmaceutical companies which have a research base have established subsidiaries in Australia. However, only a few of these organizations carry out basic research in the country, and those who do have a scientific presence here concentrate on development rather than pure research. Carrying out research and development in Australia is far less expensive than in the United States, for example. However, in preference to establishing research facilities in Australia, many organizations nowadays choose to fund universities and research institutes in their own research programmes. In human terms, Australia has no shortage of highly qualified scientists, and the quality of preclinical and clinical research is of the highest order.

When it comes to clinical trials, Australia is a favoured location for studies, on the basis of costs and medical expertise. This has come about through close collaboration between the industry as a whole, and independent research centres, including hospitals, universities and specialised research establishments.

Significant breakthroughs in Australian research in recent years have been the "gene shears" project, and the development of relaxin. In each case, Australian researchers can claim credit for the initial

work, although support for the advanced stages of many developmental programmes has to originate overseas. While these two projects, among others, have created interest in the broader scientific arena, there are a number of other promising new projects being undertaken, both within the Australian pharmaceutical industry, and in organizations funded by it.

A notable recent development within the Australian pharmaceutical industry has been the burgeoning of the regulatory and clinical functions. Regulatory affairs specialists and clinical research associates have proliferated, whereas the headcount in a number of other functions in the local industry has declined. Australia has seen a trend towards 'de-layering', which has led to flatter reporting structures in many organizations. Middle management functions in many sales and marketing departments have disappeared, with a need for marketing executives to become better negotiators who see international business development as a genuine opportunity.

There has been a trend away from management of Australian subsidiaries by expatriates from Head Office, and the local industry is now run by managers who combine familiarity with the local marketplace with an understanding of international issues which affect Australia. In an industry with a commitment to self-regulation, there is widespread participation in continuing education among pharmaceutical executives, and high levels of tertiary education can be seen in all functions, from sales, through to administration, production and general management. In scientific functions, the industry has continued to attract local graduates of the highest calibre, many of whom have benefited from overseas experience.

Conclusion

The Australian pharmaceutical industry has emerged from the tough economic climate of recent years, and the future outlook is generally positive. Standards which operate in all disciplines within the industry have now achieved parity with the highest international requirements. The local industry is well staffed by executives who are conscious of the need to compete internationally, and who are capitalizing on the country's developing ability to penetrate foreign markets. □

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1. Australian Pharmaceutical Manufacturers Association — Annual Report 1992, 1993 (APMA Inc., Sydney).
2. *Scrip Review* (APJB, London, 1993).
3. *Australian and New Zealand Biotechnology Directory 1993* (Australian Biotechnology Association Melbourne, 1993).